A STUDY ON THE FORM IN WHICH CAROTENE AND VITAMIN A OCCUR IN HUMAN BLOOD PLASMA.

BY

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There is a great number of papers published on carotene and vitamin A, dealing with various questions, such as chemical constitution, synthesis, methods of determination, estimation in various tissues and fluids under various conditions, metabolism and so on. But the number of papers on the state in which these two substances occur in nature and especially in human blood is relatively small.

Some time ago I was faced with the problem of explaining why vitamin A, a substance regarded as fat soluble, is found in urine, a watery medium, in certain pathological conditions. A close look at the problem showed an interesting feature. In almost all cases in which vitamin A could be found in urine, protein and lipoids could also be detected. (1) Similarly other workers have reported the presence of cholesterol in pathological urines, excreted by persons suffering from the same illnesses, as are accompanied by vitamin A excretion in urine (2). The idea that urine is a concentrated filtrate of blood (Cushny) suggested the advisability of trying to find out first, in what state vitamin A exists in blood, in the hope that this question being answered, the second, that of vitamin A in urine, might find an easier explanation. Since blood contains besides vitamin A, a group of closely related substances the

carotenes, my interest was naturally extended to these. As carotene or vitamin A have never been found in the red blood corpuscles, the research was limited to blood plasma or serum.

About thirty years ago Palmer (3) expressed the view that carotene exists in serum as a compound with protein. He says:-

> "The studies made by Palmer and Eckles in regard to the transportation of the carotin and xanthophyll in blood of the cow showed, that the carotins are carried as a water soluble compound of the albumin of the serum, while the xanthophyll, which is present in relatively small proportion, is carried by the fat of the blood.
>
> Probably the most striking demonstration of this is seen in the failure to extract the pigment from either the fresh or dessicated serum (plaster of Paris) with pure ether (free from alcohol), while addition of alcohol to the serum or to the plaster of Paris mass, sufficient to coagulate the proteins, will liberate the carotin so that it may be readily extracted with petroleum ether, the result being identical with the extraction of carotin from 80%-65% alcohol with this solvent."

Palmer had also pointed out that different sera behaved differently, and showed that two different sera (cow and hen) may not behave in the same way towards the same solvent (ethyl ether):-

> "Whereas it is possible to extract only traces of carotin from cow serum, the entire pigment of the hen serum was found to be readily extracted by this solvent from the fresh as well as from the desiccated serum. Both the hen and the cow serum were similar in their failure to give up their respective pigments to petroleum ether or carbon bisulphide,"(ibid.)

W,d. Bergh & Snapper (cit. by Palmer) (4) have shown that:-

> ".....the liprochrom of cattle serum is precipitated with the proteins when two volumes of 95% alcohol are added to one volume of serum, while bilirubin..... in the supernatant fluid, when the precipitated proteins are centrifugalised."

V.d. Bergh and Muller (5) assert that neither carotene nor xanthophylls can be shaken out of serum with ether. They believe that the pigments are always in a colloidal solution in plasma or serum.

Palmer (6) agrees with this view in so far as

carotene in ox and horse serum is concerned and at times in human serum. He believes, however, that in all probability a double colloidal phenomenon is involved in these cases, i.e. first, a colloidal adsorption of carotene by albumen and second, a colloidal solution of albumen in plasma. He states also that in human serum there are instances when the whole of the pigment may be extracted by ether. In his book (6) he quotes a case of an adult where on the first examination it was impossible to extract the pigment with ether, but on the second examination it was readily extracted by the same solvent. He ascribes this to the influence of diet. "the diet may influence the manner in which carotin is carried by human blood."

He does not give any proof or confirmation of this nor does he state the conditions at which his extractions have been made (pH of plasma, volume of solvent, time of shaking.) *

Pett and La Page (7) give the following statement:-

> "The nature of the complex binding vitamin A is of some interest and preliminary work with fractional precipitation suggests that it may be serum globulin. An alchoholic precipitate

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* In no case, among several hundreds of extractions carried out in this laboratory did I succeed in extracting all the carotene from plasma or serum at the ordinary pH of plasma to which anticoagulant has been added, i.e. pH between 7.2 to 8.0.

> of one blood sample showed 400 I U.
> in the precipitate and only 25 I.U.
> in the alcohol."

There is a whole literature concerning the protein symplex in rhodopsin but practically none, except that already quoted, dealing with the state of vitamin A in serum and our knowledge of the state of carotene in blood is derived mainly from the papers by Palmer and al., which give very unsatisfactory evidence.

This state of knowledge of the subject fully authorises Drummond and MacWalter (8) to express the following opinion.

> "Actually our knowledge of the physical conditions of carotene occuring normally in the blood, tissues, fluids and organs of the body is very meagre. On the fact, that the pigment cannot be always extracted by direct treatment with solvents such as ether, Palmer has based his view that it may be present in the form of a complex with proteins (carotoalbumins). The force of this argument has been weakened by V. d. Bergh and Muller having demonstrated that similar behaviour may be shown by simple aqueous colloidal suspensions of

carotene, an observation we have ourselves repeatedly confirmed."

In the same work Drummond tells that carotene may be absorbed from the intestine in the form of a water-coluble diffusible complex with bile acids and that the absorption from the intestine is to a large extent dependent on the presence of the bile acids. According to Drummond a complex of carotene with desoxycholic acid can be readily prepared. Unfortunately he does not give any technical details concerning these complexes with cholic acids, neither does he give any experience indicating the existence of such complexes in the organism.

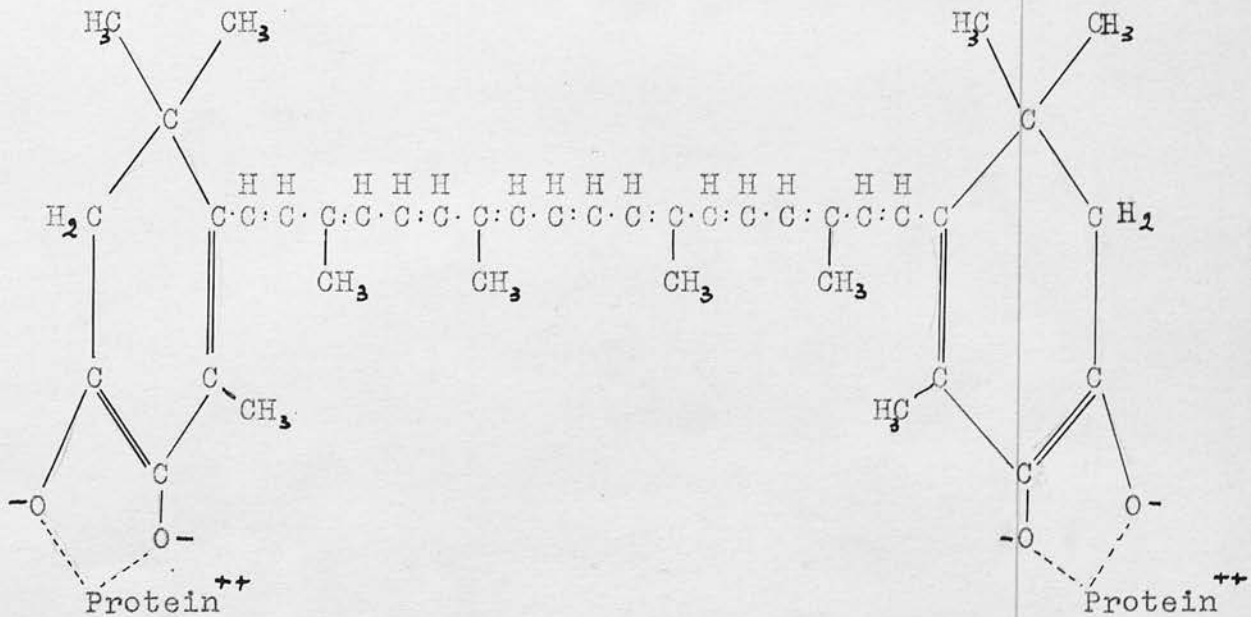
Kuhn and Bielig (9) have published in "Berichte" a paper on the symplex of lycopene with albumen in tomato. This symplex is a loose one and the pigment, which normally cannot be extracted with organic solvent is easily extracted after the precipitation of the albumen. This work is not available but is quoted by K. H. Mayer (10).

Lovern and Morton (11) consider that:-

"There is evidence that vitamin A may form a complex with protein as do certain carotenoids (egz. astacene). Such a complex exists in visual purple and probably also in liver". (Edisbury et all.)

"Rhodopsin, the pigment of the visual purple of the eye, is likewise a caretenoid proteid. According to Brunner, Baronie and Kleinau it contains β-carotene (12).
It was shown by Wald on the other hand, that vitamin A or one of its progenitors plays a role here (13).....

Ovoverdin, the colouring matter of lobster eggs is also a proteid of astaxanthin and albumin which is probably basic; Kuhn and Sörensen (14) give the following schematic formula for it;



....." K. H. Mayer (10).

Karrer and Straus (15) have examined colloidal solutions of α and β-carotene in water, and find that:-

(a) Colloidal solutions of α-carotene show a distinctly different spectrum from that in organic solvents. This difference is even greater when the solution contains Na-cholate;

(b) These colloidal aqueous solutions of α-carotene are very easily oxidizable, especially in higher temperatures and in light. Na-cholate diminishes greatly the sensitivity towards oxidation;

(c) The colloidal particles of β-carotene show a much less hydrophilic character in comparison with α-carotene and therefore a greater tendency towards precipitation. Also the sensitivity towards light and temperature as regards oxidation is much smaller here;

(d) Ascorbic acid and lecithin are potent antioxidizing agents protecting carotene in colloidal solutions.

(e) In the presence of albumen a colloidal solution of carotene looses its lipochromic character and behaves like a chromoproteid, being no longer soluble in organic solvents but soluble in water. After the precipitation of albumen, with the usual protein precipitating agents, the

whole of the carotene is found in the precipitate. The spectrum (visible) showes no change. The colloidal solutions containing lecithin (no albumen) have a different spectrum.

(f) The behaviour of colloidal solutions of carotene containing both albumen and lecithin depends on the relative amounts of these two substances present. In the presence of little lecithin and an excess of albumen the solubility corresponds to that of a chromoprotein (solubility in water); when however there is an excess of lecithin, the symplex becomes insoluble in water but shows solubility in organic solvents. In the case when the symplex is soluble in water it is possible, after precipitation with ammonium sulphate, to re-dissolve the precipitate in water. The whole of the pigment goes into solution. The sensitivity of the symplexes containing albumen and lecithin towards light is much smaller than that of those containing albumen only.

(g) These artificial symplexes containing protein and lecithin show characteristics similar to those combinations of carotene and protein, which according to Palmer exist in serum.

The reader will see that not very much material is available for formation of a clear picture of the state in which carotene and vitamin A occur in nature and especially in human blood

plasma or serum. There is a number of suppositions and assumptions but a great lack of experimental evidence.

The work to be described in this thesis was done in an attempt to shed more light on the question implied in the title.

METHODS

METHOD OF EXTRACTION.

The routine method was to shake one volume of plasma or serum with two volumes of peroxide free ethyl ether[*] vigorously by hand for ten minutes, in a separating funnel the pH of the extracted solution being previously measured or adjusted to the required value. Three extractions usually were made.

To the residue, the last ether layer having been separated, one volume of ethyl alcohol[+] was added and then two volumes of petroleum ether 40°-60° and the system again shaken by hand for ten minutes, three extracts being made.

From the combined extracts the ether was distilled off completely on a water bath at about 45°C in an atmosphere of carbon dioxide. The residue was taken up into 50 cc petroleum ether quantitatively and the petroleum ether solution

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[*] Ether was purified from peroxide form by treatment with potassium iodide and citric acid and then removing the liberated iodine by treatment with sodium thiosulphate, 30 gm. kI, 20 gm. citric acid and 30 gm. sodium thiosulphate being used for a winchester of ether.

[+] Alcohol was purified from aldehydes by method of F. L. Dunlop T.A.Ch.Soc. 28, 395, 1936, quoted by Donald W. MacArdle in "The Use Of Solvents in Organic Chemistry London 1926 p. 71.

washed with 25 cc. 3% potassium hydroxide and twice with 50 cc water. It was then dried over ahydrous sodium sulphate, and after a few hours, the petroleum ether was distilled off until about 3 cc, remained. This was transferred quantitatively into a 10 cc standard flask and made up to the mark with petroleum ether. On this solution the estimation of carotene was made.

In case of direct petroleum ether extractions, the combined extracts were distilled down to 50cc. in CO_2 atmosphere then washed and further treated as above.

The transfer of extracts from ethyl ether to petroleum ether was done in order that all carotene estimations should be made in the same solvent. On the other hand ethyl ether was the only solvent tried which did not give emulsions during shaking of plasma, without previous precipitation of protein. Emulsions were actually made sometimes at very low pH even with ether. In such cases centrifuging usually, but not always helped to separate the layers. In most cases at pH not too low (down to about pH 4·7) separation of the layers took place by itself without any help. Moving in the alkaline side of the pH scale did not cause emulsions being formed.

For estimation of carotene, vitamin A,

cholesterol and lipoid phosphorus, a photoelectric absorptiometer type "Spekker", made by Adam Hilger L.T.D. London provided with two photoelectric cells, was used. The arrangement of the photoelectric cells, diaphragms, lamp and the cell with the coloured liquid under investigation is shown diagramatically below. (Taken from a leaflet issued by Messrs. Adam Hilger Ltd.)

A 100-watt projector lamp A mounted in the cylindrical lamphouse in the middle of the instrument, and run from the electric mains supply, is the source of light. A lens B mounted in the housing to the right of the lamp forms a parallel beam of light; the beam passes on through the specimen and then falls on another lens C which forms an image of the lamp filament on a photocell D (which we shall refer to as the "indicating cell") mounted at the right-hand end of the instrument. A calibrated variable aperture E is mounted immediately in front of the lens system, and enables the intensity of the light falling on the photocell to be varied

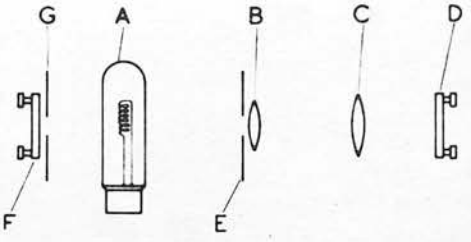
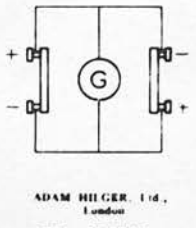


Fig. H 454a.



ADAM HILGER, Ltd., London

Fig. H 454b.

by known amounts. Since there is an image of the filaments on the cell, there is no change in the cell area illuminated when the aperture alters. The scale associated with the aperture is so calibrated that, if R is the reading corresponding to a degree of opening such that the amount of light transmitted is $1/a$ of that admitted when the aperture is fully open, then $R = \log a$. This function (known as density) has been chosen because it is approximately linear with the concentration of a solution.

Light from the lamp also falls on a photocell F mounted in the housing to the left of the lamp. The amount of light falling on this photocell (which is protected by a 1 cm. water cell) can be varied, by means of an iris diaphragm G mounted in front of it. We shall refer to this photocell as the "compensating cell." The two photocells are connected in opposition across a galvanometer so that, when the photoelectric currents given by the cells are equal, the galvanometer shows zero deflection.

The cell containing the coloured liquid had a capacity of about 8cc. In the course of the work it was replaced by another one of 6cc capacity produced during the war. The former was round shaped the latter square shaped. This, of course, did not affect the results adversely, but necessitated recalibration.

For measuring the intensity of the yellow colour of carotene blue filters No. 6 of the set provided have been used.

For the blue colour of the Carr-Price reaction as well as for the blue colour produced by molybdate in phosphorus estimations and the greenish-blue colour of the Lieberman-Burchardt reaction for cholesterol, red filters No 1 of the set were used. Calibration curves were prepared in all cases.

Carotene calibration curve

For this β-carotene obtained from B.D.H. was used. A known amount of β-carotene was dissolved in a known volume of petroleum ether 40°-60°. From this solution, usually containing 50 γ per 1cc (by dissolving 25 mgr. in 500cc petroleum ether) another solution containing 5 γ per 1cc was made.

To obtain the curve 1, 2, 3, 4, and so on cc.

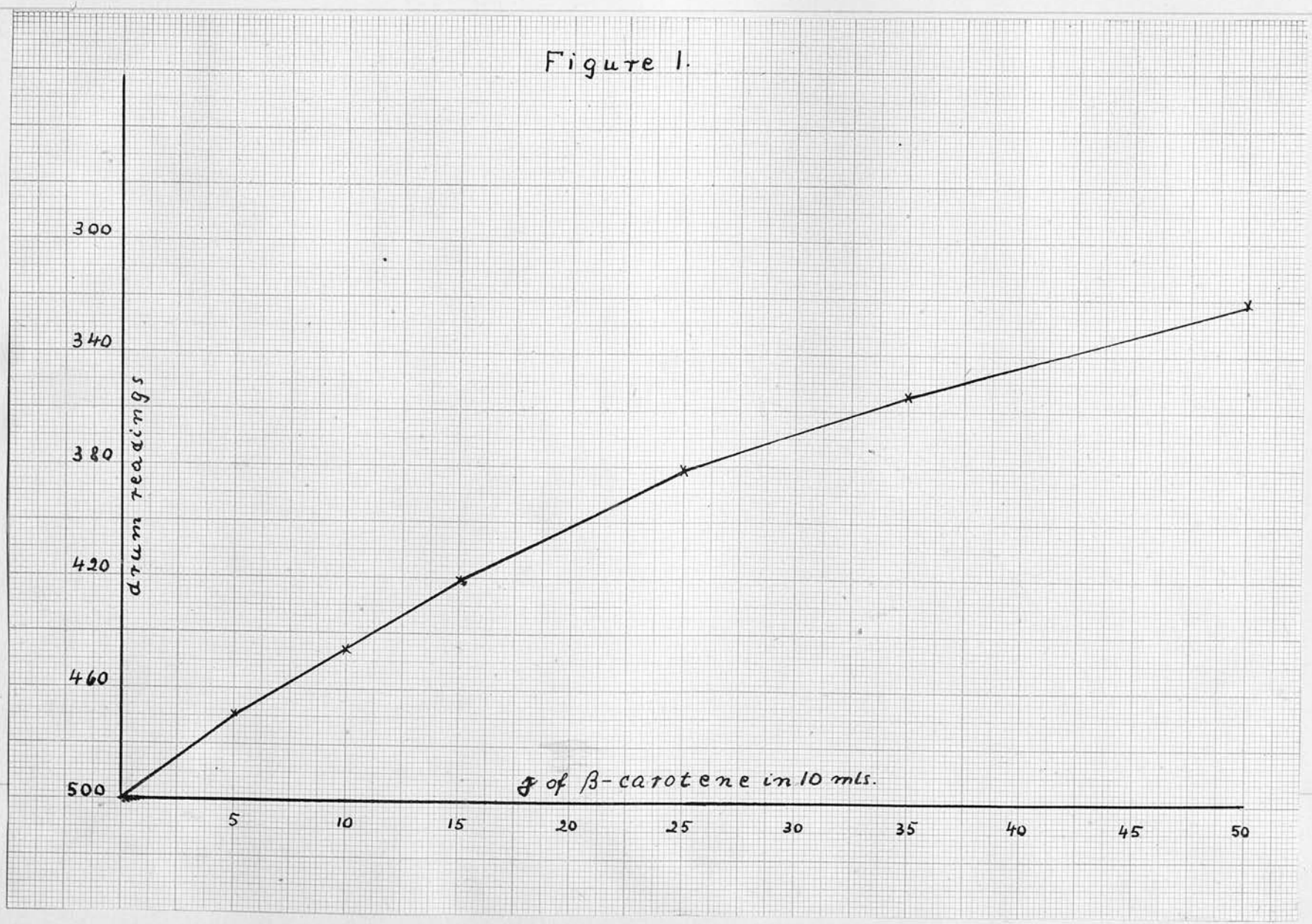
of the last solution were made up to 10cc with petroleum ether and the intensity of the yellow colour measured.

Blank was made with petroleum ether in the cell, and the drum set on ·500. The intensity of the colour has been measured not by the deflection values of the galvanometer but by the drum values obtained by moving the drum to bring the deflected galvanometer pointer to the central position.

For making the blank with red filters, the drum is set on 1·00. This is done in order to work at the same sensitivity of the instrument in both cases.

By plotting the concentrations of β-carotene in Io cc against the drum readings a line was obtained, which was never straight. It always tended to bend slightly down with increasing concentrations(. Figure 1.).

Figure 1.


300
340
380
420
460
500
drum readings
5
10
15
20
25
30
35
40
45
50
g of β-carotene in 10 mls.

The concentrations of β-carotene in 10 cc of petroleum ether expressed in γ and the corresponding drum readings are given below.

γ of β-carotene in 10 cc ptr. ether.	Drum readings
5	470
10	446
15	421
25	382
35	355
50	322

Vitamin A calibration curve.

The 2-naphthoate of vitamin A was used as a standard.

This crystalline substance was obtained from B. D. H. in sealed tubes. 1 gm. of vitamin A naphthoate corresponds to 2225000 I.U. vitamin A. (16)

It is usually dissolved in arachis oil stabilised with 0.01% hydrochinone. Since, however arachis oil is rather difficult to pipette the naphtoate may be dissolved directly in chloroform and then diluted to will. The naphthoate solution in chloroform is unstable and marked decomposition takes place within few hours. Therefore, the curve must be prepared at once after the naphthoate had been dissolved in chloroform. In arachis oil it may be kept safely for weeks.

10 mgr. of the naphthoate, corresponding to 22250 I.U. was dissolved in 100 cc dry chloroform. 1 cc of this solution corresponding to 222.5 I.U. was made up to 5 cc with chloroform. From the latter solution 0.5 cc, 1 cc, 1.5 cc, 2 cc and so on were taken and again made up to 5 cc with chloroform. 0.5 cc of each of those solutions was taken and added to 5 cc of the Carr-Price

reagent. Corresponding drum readings were taken.

I.U. vitamin A in 5 cc	drum readings
22.25	968
44.5	938
66.75	906
89.0	875
111.25	844
178.0	750

A straight line was produced by plotting the drum values against the I.U. of vitamin A in 5 cc. chloroform solution(Figure 2.)

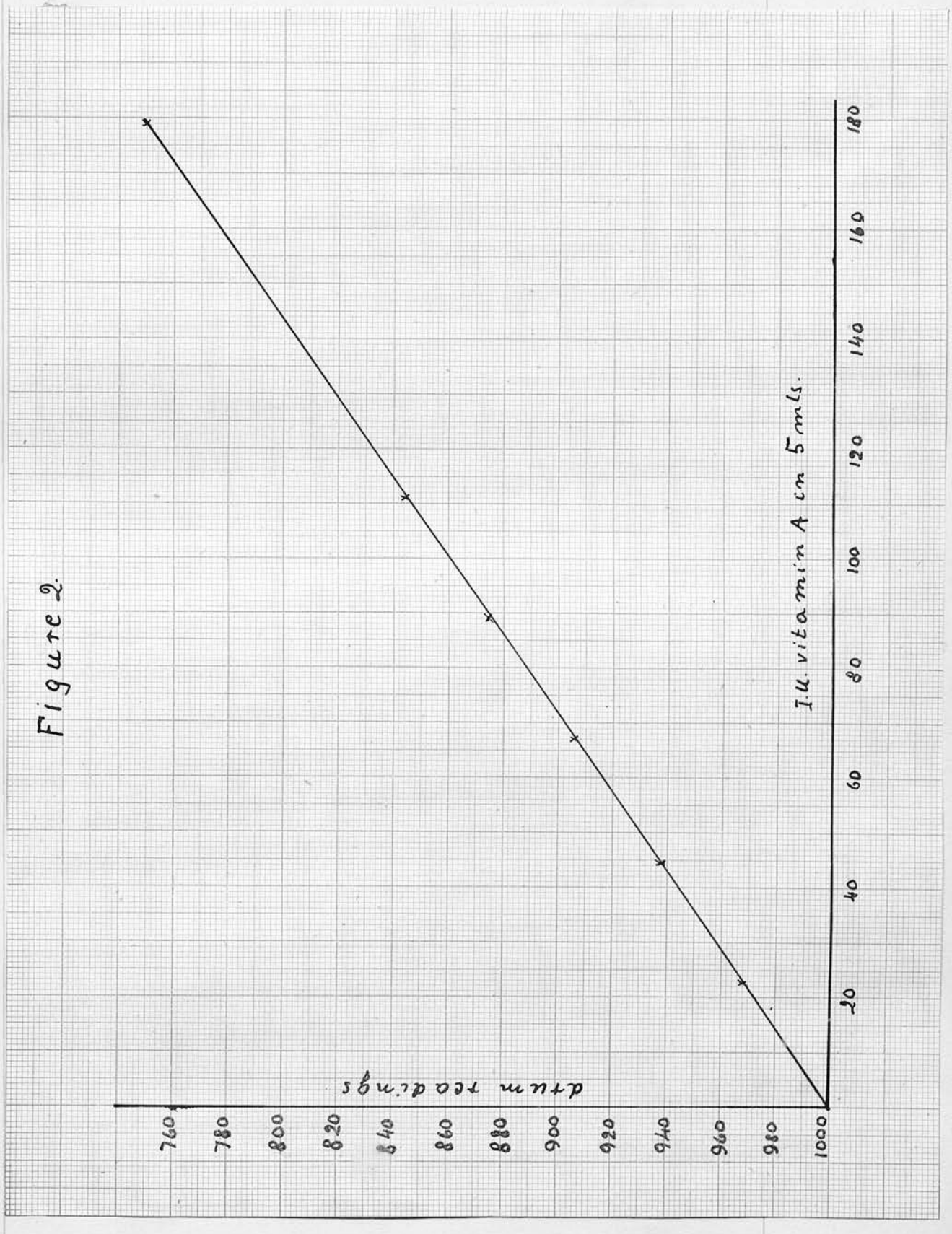

760
780
800
820
840
860
880
900
920
940
960
980
1000
datum readings
20
40
60
80
100
120
140
160
180
I.U. vitamin A in 5 mls.

Figure 2.

The blue Carr-Price colour obeys the Beer's Law better than does the yellow of carotene.

Expression of β-carotene in I.U. of vitamin A.

The petroleum ether solutions of β-carotene as for preparation of carotene calibeation curve (10 ml.). were transferred quantatively in 25 ml. distilling flasks and the solvent distilled off in carbon dioxide atmosphere on water bath at 45 C.

The residue (β-carotene) from each flask was taken in 5 ml. dry chloroform. 0.5 ml. of the latter solution being added to 5 ml. of Carr-Price reagent and the intensity of the blue colour produced measured.

The number of I.U. vitamin A corresponding to the known amounts of carotene expressed in γ were plotted against each other. A straight line was obtained.

carotene	I.U. vitamin A
15	8.5
25	14.5
35	20.0
50	29.0

(Figure 3.)

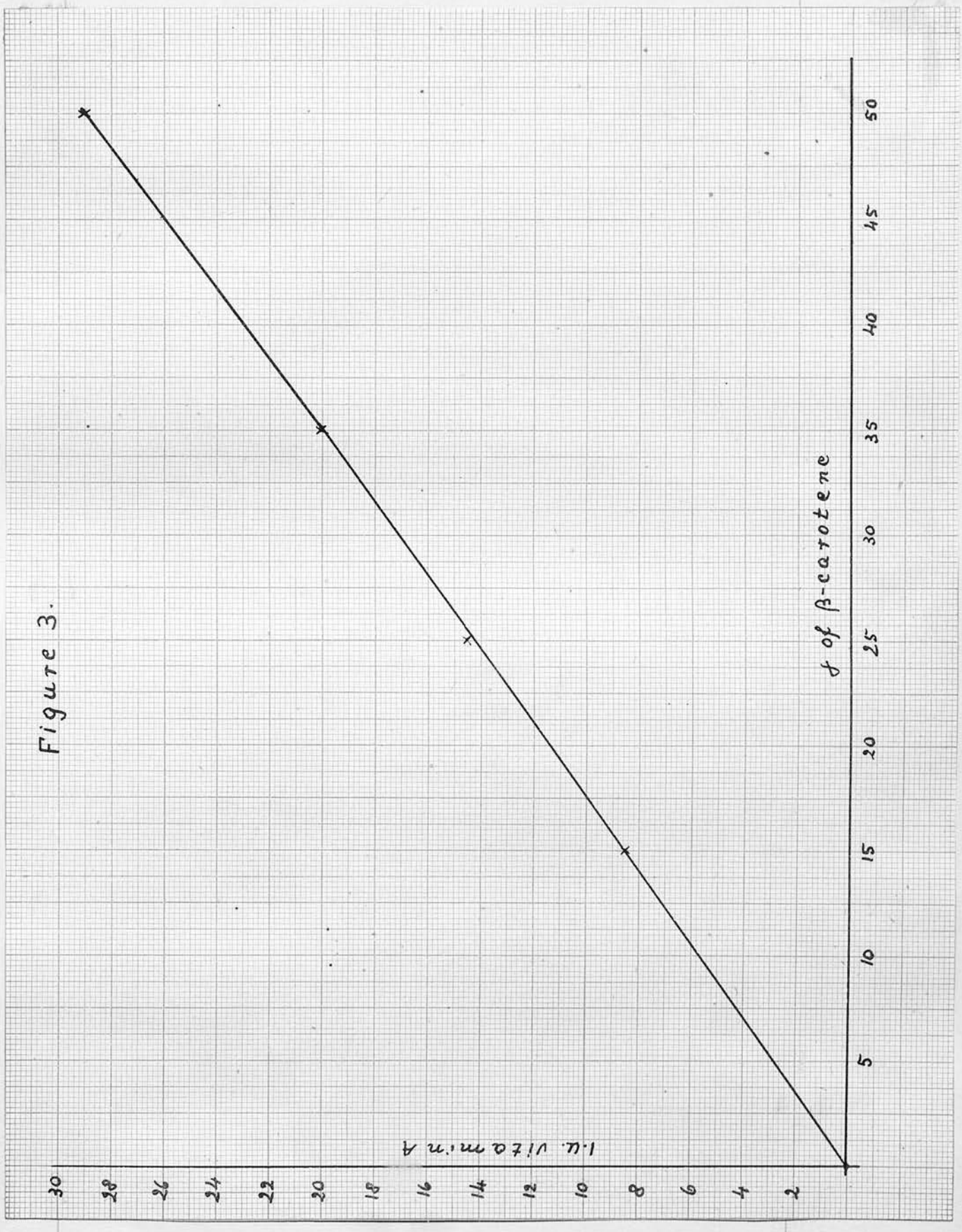

I.U. vitamin A
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
5
10
15
20
25
30
35
40
45
50
% of β-carotene

Figure 3.

Estimation of carotene and vitamin A in plasma Extracts.

Petroleum ether extracts containing carotene and vitamin A after having been washed dried and filtered, were distilled (at 45°C in CO_2 atmosph.) down to about 3 cc and then transferred quantitatively into 10 cc standard flasks and made up to the mark with petroleum ether. The intensity of the yellow colour was measured on these solutions.

This having been done, the petroleum solution was transferred quantitatively into 25 cc. distilling flask. The solvent distilled off on a water bath at 45°C in carbon dioxide atmosphere. To the residue (an oily drop) 2 cc dry chloroform were added and transferred quantatively into a standard 5 cc. flask, the distilling flask being washed three times with 1 cc chloroform. From this 5 cc chloroform solution 0.5 cc. was taken for the Carr-Price reaction. When a very small amount of vitamin A was expected the residue was dissolved in 2 cc chloroform. In case of greater concentration, beyond the range of the calibration curve the solution had to be diluted appropriately.

Great care must be taken that the final chloroform solution is completely dry, otherwise a cloudiness or turbidity will be produced due to

the hydrolysis of antimony trichloride. This turbidity may be a source of great error as the photoelectric absorptiometer, measuring the amount of light transmitted is also sensitive towards turbidity.

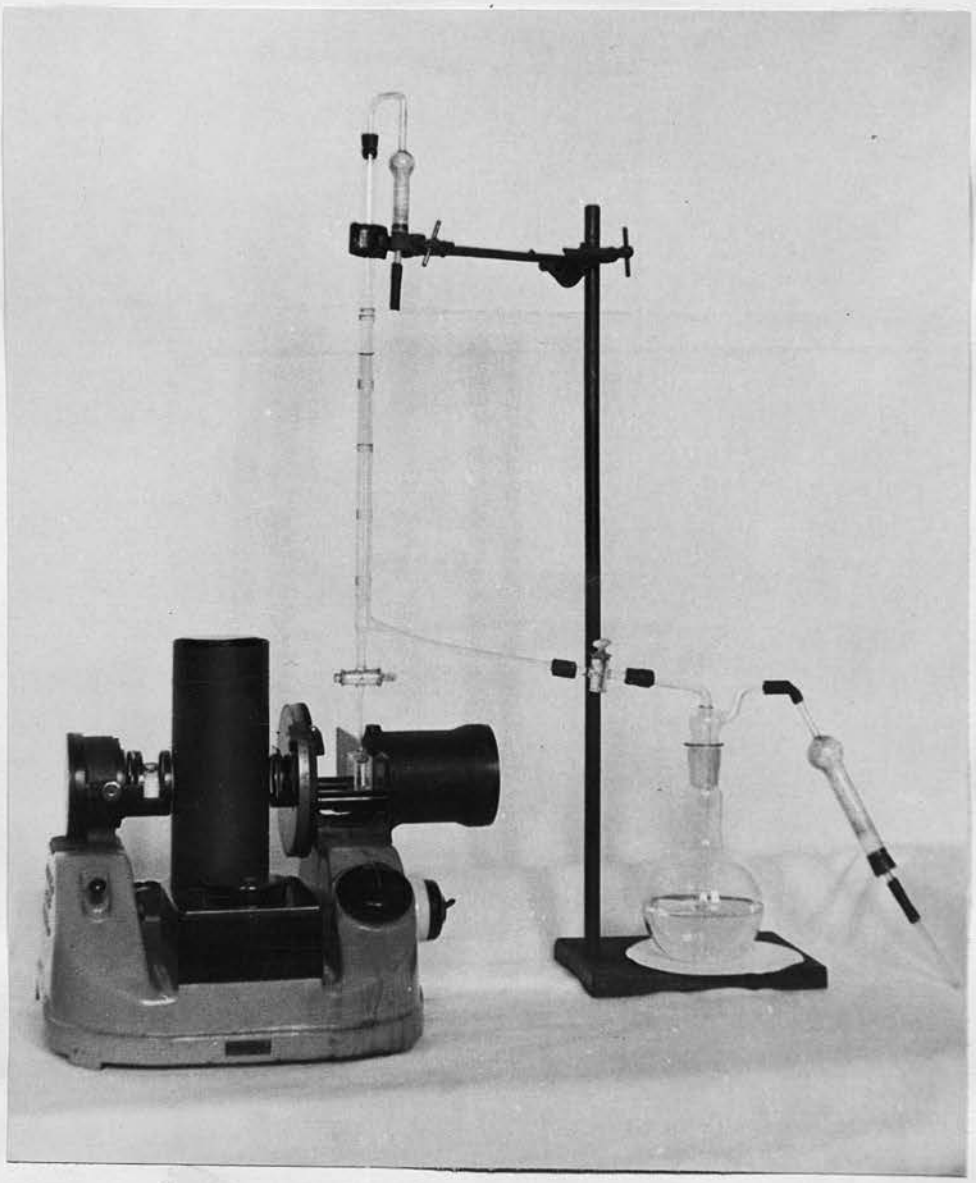
To bind the possible traces of water, a few drops of acetic anydride may be added.

The blue colour produced by Vitamin A and the Carr-Price reagent is rather unstable and reaches its maximum within about 20 seconds. The measurement must therefore be accomplished during that time.

The question of keeping the Carr-Price reagent, so sensitive towards traces of moisture, and the way of adding it into the cell of the colorimeter has caused much discussion and many devices have been proposed (17). It seems to me that any arrangement which provides means of storing the reagent without the access of moisture and, at the same time, allows a rapid discharge of a stated amount of the reagent into the absorptiometer cell, is good.

My arrangement involves the use of a thirty ml. burette with a side glass tube fixed to it, a glass-jointed wash bottle and a two way stop cock. (Figure 4.)

Figure 4.



The burette, by its side tube is connected with the wash bottle through a stop cock with the help of two short pieces of pressure tubing.

The antimony trichloride reagent occupies the wash bottle. It is kept in darkness by covering the bottle with a piece of dark cloth and putting in a carboard or wooden box. The outlet of the wash bottle is provided with a soda lime tube and anhydr. calcium chloride tube. The upper end of the burette has a similar tube containing calcium chloride only.

The wash bottle rests on the base of an ordinary lab iron stand, the burette being clamped to it.

When the arrangement is not in use, the stop cock is shut. The calcium chloride tube prevents any moisture from reaching the inside of the wash bottle.

Before use the stop cock is open and air blown by mouth inside the wash bottle through the soda lime, calcium chloride tube. Carbon dioxide and water vapour are thus held in the tube. The increasing pressure inside the wash bottle drives the reagent in the burette. The stop cock is now shut to keep the liquid in the burette. To help the control of the outflow of the reagent, little strips of paper are fixed on the burette

dividing it into required volumes.

After the "blank" has been done with the antimony reagent in the cell, 0•5 cc of the examined solution is poured in the cell and then, in a matter of three seconds, 5 cc of the Carr-Price reagent. The rapid flow does the mixing.

I found it equally satisfactory to pour the antimony reagent first in the cell, do the blank, and add the solution under examination from a 0•5 cc pipette with a relatively large outflow. Mixing is quite good if the outlet of the pipette is kept about 2-3 cm. above the level of the reagent in the cell.

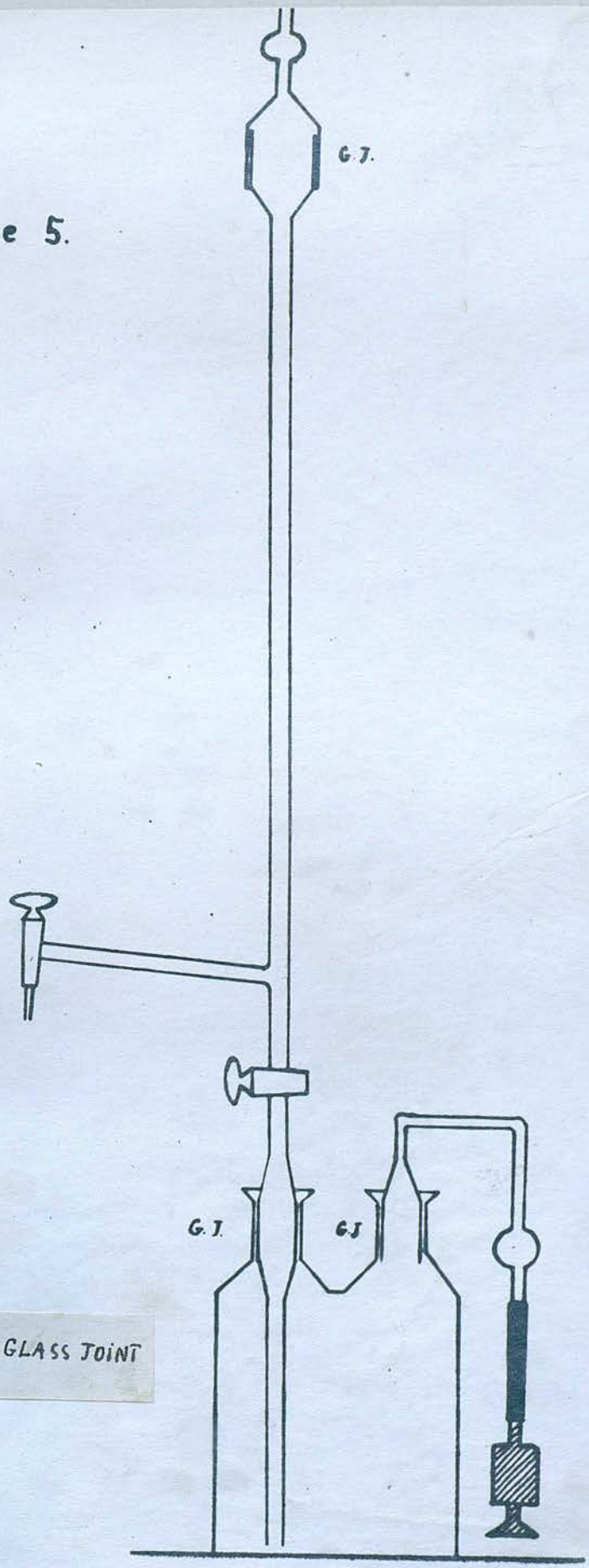
There was no difficulty with the reagent being hydrolyzed in nozzle of the burette except when the weather was very damp. A heater placed near would remove the trouble.

The short pieces of pressure tubing connecting the stop cock with the burette and the wash bottle are exposed to the action of chloroform which destroy rubber. It takes however a few months till they have to be exchanged, especially when the ends of the glass tubes inside the rubber tubing are close together.

The length of the side tube of the burette was in my case twentyfive cm. It should be long

enough to allow free access to the cell of the absorptiometer. The tube should be slightly bent down so that any unused reagent might be easily run back to the bottle. An ideal arrangement would be one with all glass joints as represented on Fig. 5.

Figure 5.



G.J. = GLASS JOINT

The cell, after pouring off of the reagent, becomes cloudy as a result of hydrolysis of the reagent. Washing and drying of the call may be done by rinsing with 10% hydrochloric acid then with water, alcohol and finally with ether. Ether is dried quickly by blowing compressed air for a while, if available. Washing and drying of the cell does not take longer than two minutes.

Chloroform used for the final solution of the extracts and for the preparation of the Carr-Price reagent was always washed three times with equal volume of water, dried over anhydrous potassium carbonate filtered and distilled, all operations being done in darkness, the first portion being discarded as usually containing some moisture. The distilled chloroform may be tested for moisture by the Carr-Price reagent which on addition of a few drops of distillate should not show any cloudiness.

The preparation of the antimony trichloride reagent was done according to (18).

Cholesterol estimation

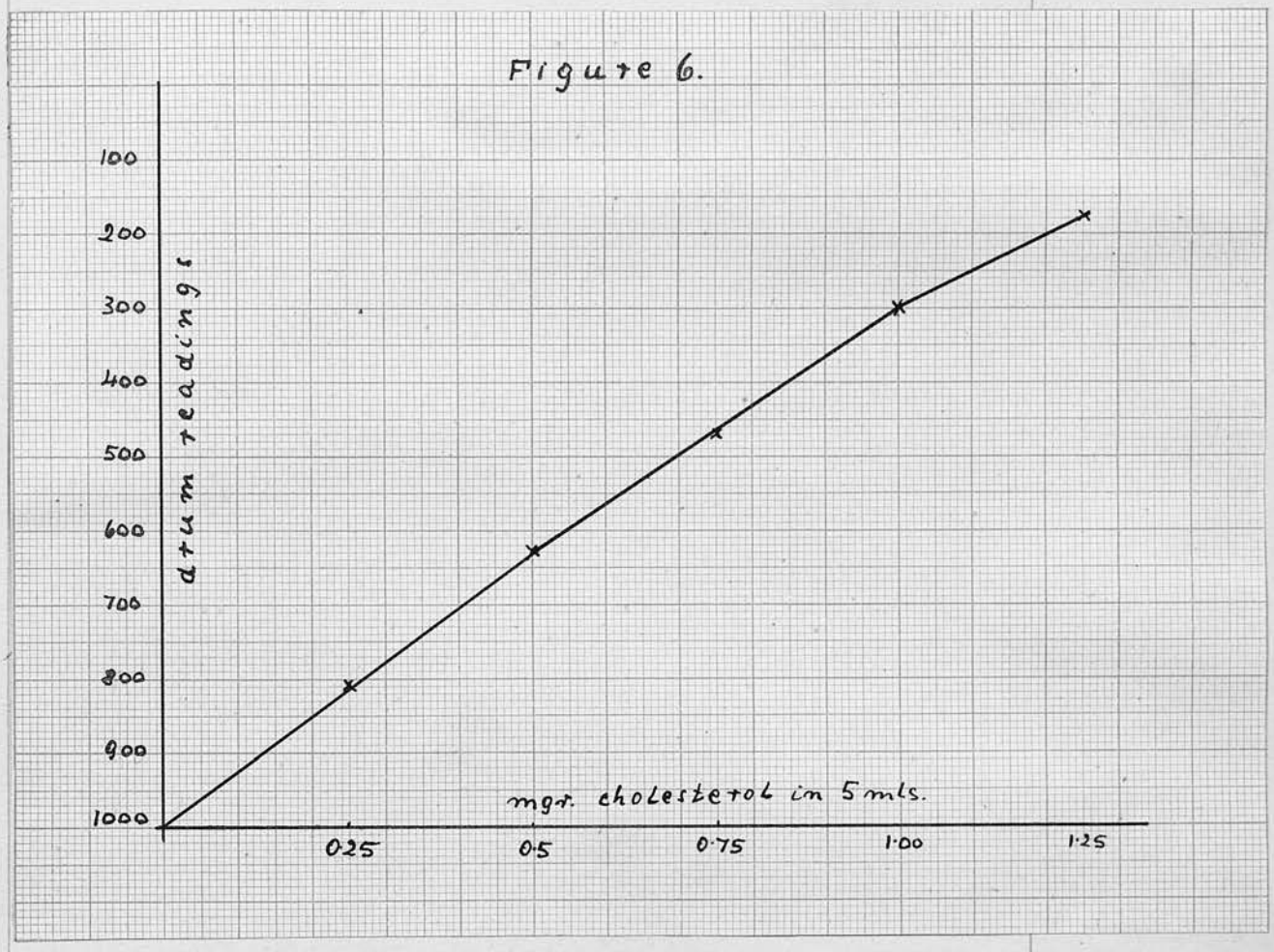
The estimations of cholesterol were done by quantitative adaptation of the Lieberman-Burchardt reaction.

Preparation of calibration curve.

25 mgr. cholesterol were dissolved 100 cc chloroform. Each cc of this solution contained 0.25 mgr. cholesterol.

1, 2, 3, 4, 5 cc of this solution were taken and made up to 5 cc with chloroform. Thus solutions of increasing concentrations of cholesterol were prepared. To each of the 5 cc chloroform colutions 2 cc acetic anhydride and 0.1 cc conc. sulphuric acid have been added and the intensity of the bluish-green colour measured after 15 minutes in the colorimeter.

By plotting the drum values against the concentrations of cholesterol in 5 cc, a curve was obtained which had a shape of an almost straight line(Figure 6.).



The concentrations of cholesterol in 5 cc and the corresponding drum readings were as follows.

mgr.cholest.in 5 cc after addition of the reagents	drum readings
0.25	808
0·5	630
0·75	470
1·0	300
1·25	178

For actual estimations in the extracts, 0•5 cc. of the chloroform solution of the extract used for vitamin A estimation was made up to 5 cc. with chloroform, then 2 cc acetic anhydride and 0•1 cc conc, sulphuric acid were added and after 15 minutes the intensity of the bluish-green colour was measured. The amount of cholesterol read from the curve had to be multiplied by 10 as one tenth of the original chloroform solution of the extract was taken.

It must be noted here that carotene, present in the cloroform solution used for the estimation of cholesterol gives also a blue colour with the reagents used to produce the bluish-green colour of the Lieberman-Burchardt reaction. This blue colour produced by carotene however reaches its maximum within give seconds of the addition of the sulphuric acid and then rapidly fades away. It therefore is not an obstacle in the estimation of cholesterol. Vitamin A also present in the chloroform solution does not give any colour with the Lieberman-Burchardt reagents.

Lipoid phosphorus estimation

To prepare the calibration curve 1.155 gm. crystalline sodium hydrogen phosphate ($Na_2HPO_4 \cdot 12 H_2O$) was dissolved in one liter distilled water. 1.155 gm. of the salt contains 0.1 gm. phosphorus. 1, 2, 3, 4, and so on cc of the above solution were made up with water to 100 cc, thus solutions of increasing concentrations of phosphorus were prepared.

To 2 cc of each of these solutions (containing 0·1, 0·2, 0·3 a s. o. mgr. phosphorus in 100 cc), 5 cc water, 2 cc ammonium molybdate solution and 1 cc stannous chloride solution were added. After six minutes from the addition of the sulphuric acid the intensity of the blue colour was measured and the corresponding drum readings noted. By plotting the drum readings by the phosphorus concentrations in 100 cc a line was produced which was practically straight (Figure 7).

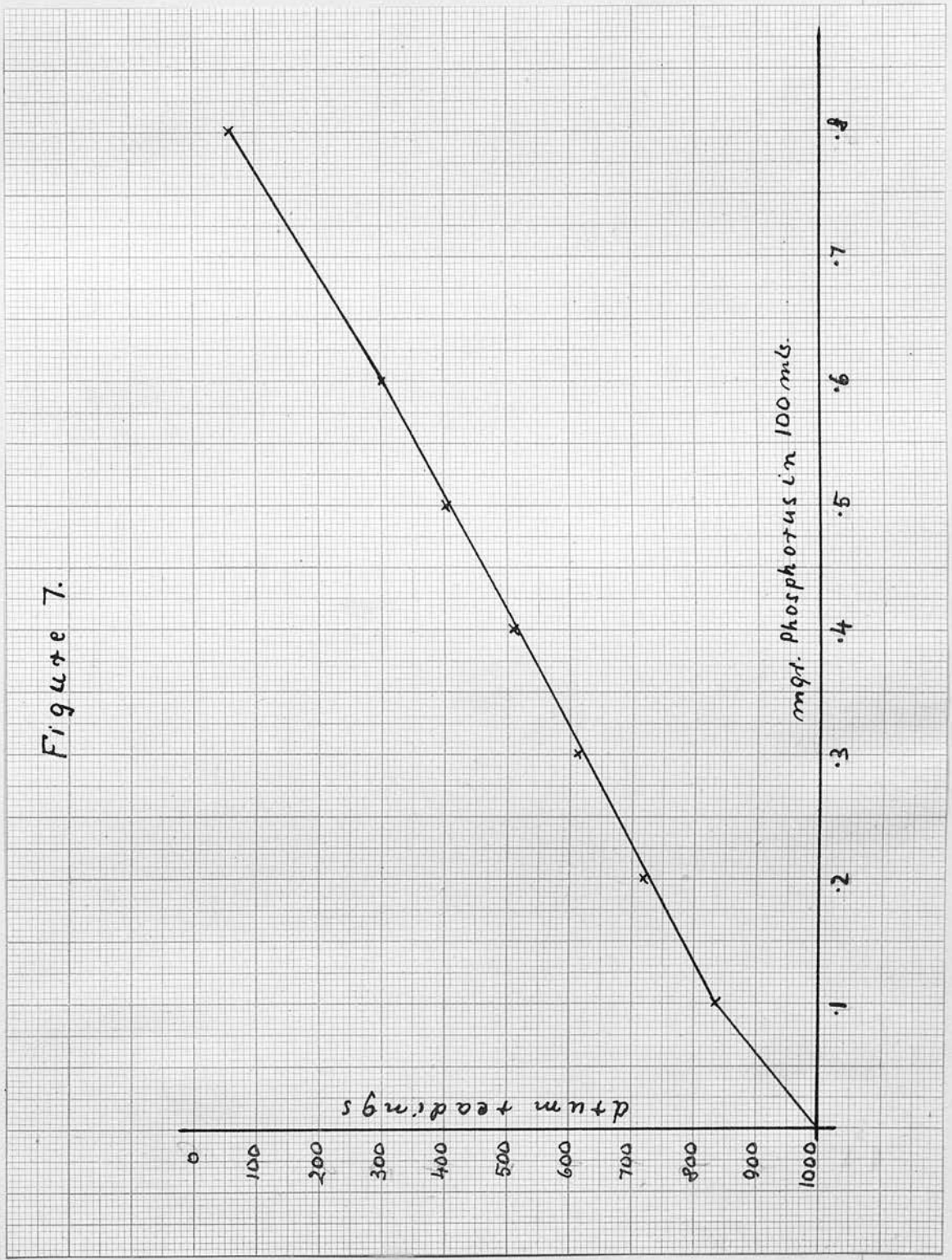

0
100
200
300
400
500
600
700
800
900
1000
datum readings
.1
.2
.3
.4
.5
.6
.7
.8
mgm. phosphorus in 100 mls.

Figure 7.

The concentrations of phosphorus per 100 cc. and the corresponding drum readings are given below.

mg. phosphorus per 100 cc	drum readings
0·1	838
0·2	720
0·3	612
0·4	510
0·5	402
0·6	302
0·8	060

The time of 6 minutes for measurement of the intensity of the blue colour after addition of stannous chloride was chosen as a result of a study of the time, in which the blue colour reaches its manimum within the range of concentrations used. It was found that 6 minutes was the average time when the maximum has been reached.

For the actual estimations of lipoid P in plasma extracts, the organic solvent of the extract was distilled down to about 10 cc. and the solution transferred quantitatively into an improvised micro digestion flask blown out of a pyrex test tube to give a bulb of about 20 cc. capacity. The rest of the solvent was now evaporated by keeping the digestion flask in hot

water in a beaker, using glass beads to ensure even boiling.

To the residue, (an oily spot), 2 cc conc. sulphuric (AR) acid were added and the flasks placed in a digestion stand. The combustion was over in 3-4 hours, a few drops of hydrogen peroxide being added at the end.

The clear, colourless solution was transferred quantitatively into 100 cc standard flask and made up with water to the mark. Two cc. of this solution taken for estimation, being treated as for the preparation of the calibration curve.

The ammonium molybdate solution was prepared according to A. P. Briggs (19) analar reagents being used.

The stannous chloride solution was prepared according to Kuttner and Lichtenstein quoted by Hawk (p. 20).

RECOVERY OF CHLOROFORM FROM RESIDUES AFTER VITAMIN A ESTIMATIONS.

When tens of estimations are being made daily, for each about 6 mls. chloroform being used, it is found that a winchester or two of the residues accumulate in a few weeks time. Taking into account the relatively high price of chloroform and war time conditions it would seem to be a waste to throw the residues away. According to the following method, most of the chloroform may be recovered.

The dark coloured chloroform containing antimony trichloride acetic anydrice products of decomposition of carotene, vitamin A and of the blue coloured compound, is first distilled by ordinary distillation. The distillate is colourless but still contains part of the antimony trichloride and probably acetic anhydride. It is now washed twice with equal volume of 18% hydrochloric acid to remove the antimony trichloride and its hydrolysis products and afterwards four times with at least equal volume of water and dried over anhydrous potassium carbonate.

Final purification is done by distilling through a fractionating colum.

E X P E R I M E N T A L.

INFLUENCE OF pH ON THE EXTRACTABILITY OF CAROTENE AND VITAMIN A FROM HUMAN PLASMA OR SERUM.

In all experiments described in this section extraction was carried out as described in the paragraph dealing with the method of extraction. Nine experiments were done, the pH values varying slightly in different experiments. In all experiments to be described in this work pH was measured by the Beckmann pH meter except of the first one of this section, in which it was judged roughly with the use of B. D. H. Universal indicator.

Results per 100 ml.of plasma expressed in gamma of β-carotene and in I.U. of vitamin A are given in the following tables:-

E = Extract, R = Residue, T = Total.

Experiment 1. Table 1.

		Caro-tene,	% of T.	Vitamin A	% of T.
	E.	8	9	61	72
I.pH 10	R.	76	--	24	--
	T.	84	--	85	--
	E.	14	16	66	68
2.pH 7.6	R.	76	--	30	--
	T.	90	--	96	--

		Carotene,	% of T.	Vitamin A	% of To.
		E. 77	91	73	75
3.pH 3		R. 7	--	24	--
		T. 84	--	97	--

Experiment 2. Table 2.

		Carotene,	% of T.	Vitamin A	% of T.
		E. 22	33	120	84
1.pH 10.5		R. 45	--	22	--
		T. 67	--	142	--
		E. 22	28	90	68
2.pH 7.6		R. 55	--	30	--
		T. 77	--	120	--
		E. 50	79	100	90
3.pH 5		R. 13	--	10	--
		T. 63	--	110	--
		E. 57	80	102	91
4.pH 3		R. 13	--	10	--
		T. 70	--	112	--

Experiment 3. Table 3.

		Carotene,	% of T.	Vitamin A	% of T.
	E.	15	24	34	33
1.pH 8.3	R.	47	--	70	--
	T.	62	--	104	--
	E.	6	9	23	22
2.pH 7.6	R.	58	--	84	--
	T.	64	--	107	--
	E.	60	100	101	87
3.pH 6.7	R.	0	--	15	--
	T.	60	--	116	--
	E.	60	100	101	100
4.pH 5.9	R.	0	--	0	--
	T.	60	--	101	--
	E.	60	90	125	96
5.pH 5.2	R.	6	--	11	--
	T.	66	--	136	--
	E.	50	89	147	93
6.pH 4.5	R.	6	--	11	--
	T.	56	--	158	--

Experiment 4. Table 4.

		Carotene,	% of T.	Vitamin A	% of T.
	E. 14	25		86	59
1.pH 8.22	R. 42	--		59	--
	T. 56	--		145	--
	E. 11	20		83	59
2.pH 7.7	R. 45	--		56	--
	T. 56	--		139	--
	E. 9	18		76	56
3. pH 7.3	R. 42	--		59	--
	T. 51	--		135	--
	E. 21	39		95	70
4.pH 6.9	R. 33	--		41	--
	T. 54	--		136	--
	E. 32	58		98	83
5.pH 5.6	R. 23	--		20	--
	T. 55	--		118	--
pH 7.7	E. 16	31		93	69
6. from	R. 32	--		41	--
pH 5.6	T. 48	--		134	--

No. 6 in this experiment was adjusted to pH 5.6 and after one minute brought to pH 7.7.

Experiment 5.

Table 5.

	Carotene	% of T.	Vitamin A.	% of T.
	E. 40	40	81	65
1. pH 9.3	R. 60	--	44	--
	T.100	--	125	--
	E. 35	40	60	48
2. pH 7.6	R. 51	--	65	--
	T. 86	--	125	--
	E. 60	60	107	83
3. pH 6.9	R. 40	--	21	--
	T.100	--	128	--
	E. 97	97	138	96
4. pH 4.9	R. 4	--	2	--
	T.101	--	140	--
pH 7.3	E. 51	50	79	62
5. from	R. 51	--	48	--
pH 4.9	T.102	--	127	--

No. 5 of this experiment was treated as No. 6 in experiment 4.

Experiment 6

In this experiment one sample was extracted at the original pH i.e. 7.6, the other at pH 4.7, the isoelectric point of serum albumen.

Table 6.

	Caro-tene	% of T.	Vitamin A	% of T.
1 pH 7.6	E. 49	37	133	55
2 pH 4.7	E.131	100	235	100

Experiment 7

Table 7.

	Caro-tene	% of T.	Vitamin A	% of T.
	E. 15	24	105	74
1 pH 8.9	R. 46	--	36	--
	T. 61	--	141	--
	E. 15	26	82	57
2 pH 7.5	R. 42	--	61	--
	T. 57	--	143	--
	E. 18	31	115	80
3 pH 6.9	R. 40	--	28	--
	T. 58	--	143	--
4 pH 5.1	E. 42	69	142	99

		Carotene	% of T.	Vitamin A	% of T.
4.pH 5.1	R.	20	--	7	--
	T.	62	--	149	--

Experiment 8.

Table 8.

		Carotene	% of T.	Vitamin A	% of T.
	E.	14	19	112	95
1. pH 7.7	R.	60	--	6	--
	T.	74	--	118	--
	E.	26	37	138	98
2.pH 6.5	R.	52	--	2	--
	T.	78	--	140	--
	E.	36	45	120	83
3.pH 5.4	R.	44	--	14	--
	T.	80	--	134	--
pH 7.6	E.	22	31	108	90
4. from	R.	48	--	12	--
pH 5.4	T.	70	--	120	--

The results of this particular experiment are not typical. There was some precipitation of plasma proteins already at original pH i.e. 7.6.

Experiment 9.

Table 9.

		Carotene,	% of T.	Vitamin A	% of T.
	E.	14	20	60	60
1. pH 7.6	R.	56	--	40	--
	T.	70	--	100	--
	E.	20	31	84	76
2 pH 6.8	R.	44	--	28	--
	T.	64	--	112	--
	E.	44	69	98	86
3 pH 5.2	R.	20	--	16	--
	T.	64	--	114	--
pH 7.6	E.	28	44	76	70
4. from	R.	36	--	32	--
pH 5.2	T.	64	--	108	--

The general results expressed as percentage

of carotene and vitamin A in the extracts are summarised below.

As total or 100% is understood the amount of substance extracted by the known methods.

Table 10.

(Figures express % carotene and vitamin A in extracts)

pH	10-9		9-8		8-7		7-6		6-5		5-4		4-3	
	c.	v.	c.	v.	c.	v.	c.	v.	c.	v.	c.	v.	c.	v.
Exp.1	9	72			16	68							91	75
" 2					28	68			79	90			80	91
" 3			24	33	9	22	100	87	100	100	89	93		
" 4			25	59	20	59	39	70	58	83				
" 5	40	65			40	48	60	83			97	96		
" 6					37	55					100	100		
" 7			24	74	26	57	31	80	68	99				
" 8					19	95	37	98	45	83				
" 9					20	60	31	76	69	86				
avrg.	25	68	24	55	24	59	49	82	70	90	98	98	86	83

c - carotene, v - vitamin A.

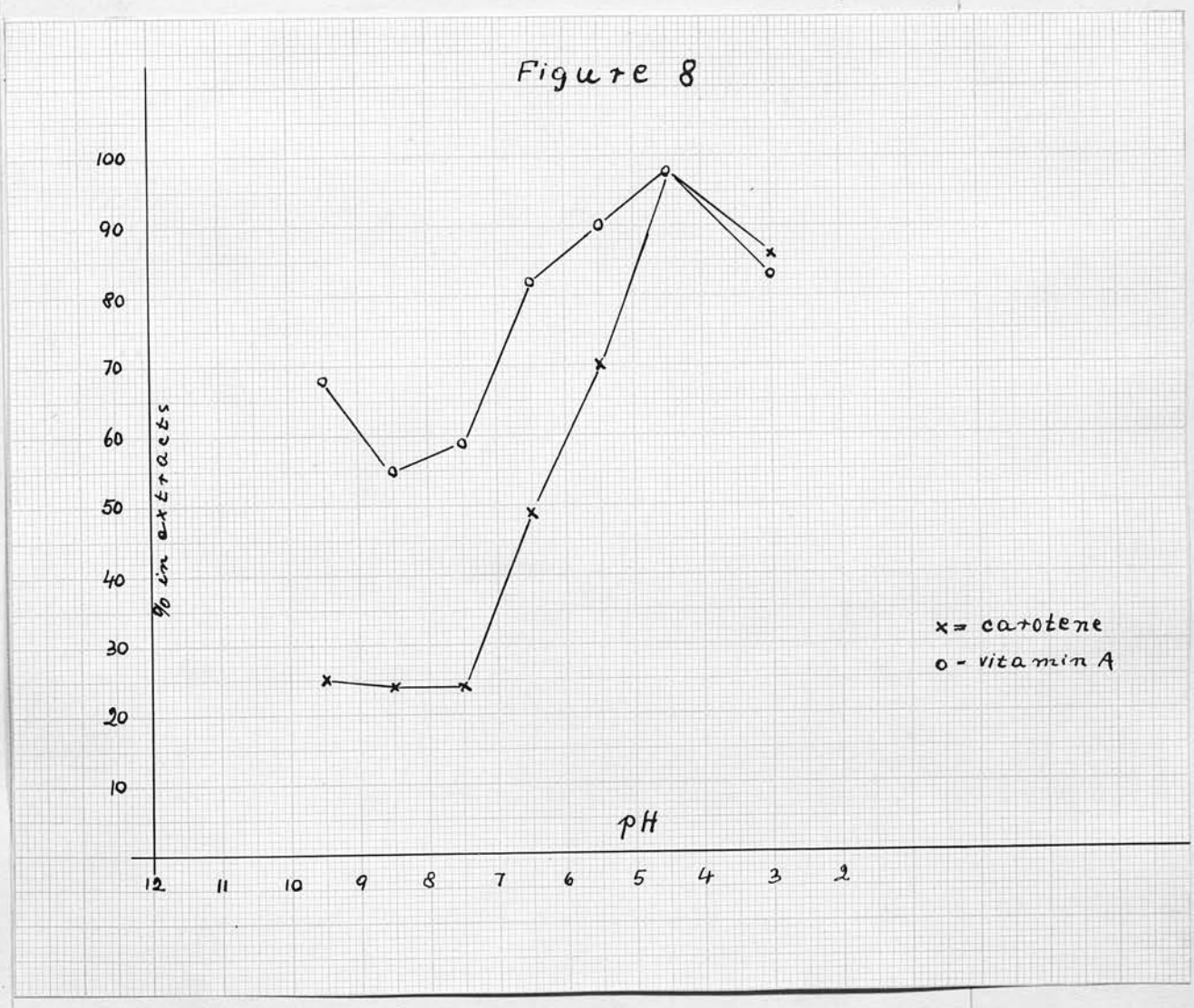
It is seen from these results that pH has an influence on the extractability of both carotene and vitamin A. 24 % carotene and 59% vitamin A are extracted at the original pH of citrated plasma (50 ml. 3.8 % sodium citrate per 450 ml. blood) which is in the region of 7.6. Moving into the alkaline side of the pH range does not produce any great change in the extractability of carotene, but that of Vitamin A increases slightly at pH between 9 and 10.

When pH is changed slightly below 7 (to about 6.8) a sharp increase in the extractablity of both carotene and vitamin A is seen (Carotene from 24% to 59%, vitamin A from 59% to 82%). With further movement to the acid side the extractability increases still further, being greatest at pH around 4.7, at that pH the iso-electric point of serum albumen, the extraction of both is practically complete. Still further movement to acid side produces a slight decrease in the extractability, Carotene from98% to 86%, vitamin A from 98% to 83%.

The curves below represent the extractability of carotene and vitamin A in relation to pH.

Fig. 8.

Fig. 8.



During these experiments one or two phenomena worthy of remark were observed. Thus the carotene and vitamin A in the extracts were always accompanied by a yellow pigment, the amount of which was roughly proportional to the amount of carotene. This pigment was washed out of the ether or petroleum ether extracts with 3% potassium hydroxide, a normal procedure in our method, and the intensity of the potassium hydroxide washings increased as the pH of the plasma moved to the acid side. It is represented below.

pH	7.6	+
"	6.8	+++
"	5.4	++++
"	4.7	+++++

This pigment gave a positive V. der Bergh reaction and proved to be bilirubin or at least to contain bilirubin.

Secondly, opportunity was taken to demonstrate that at low pH carotene and vitamin A do not undergo autooxidation at a rapid rate during the first two hours.

Two samples of fresh human plasma were acidified to pH 3. One was extracted immediately with 80 ml. ethyl ether the other was left standing for two hours and then extracted in the same way.

Carotene and vitamin A was estimated in both cases.

	Immediate extr.	Delayed extr.
Carotene	26	27
Vitamin A	117	117

THE INFLUENCE OF FREEZING ON THE EXTRACTABILITY OF CAROTENE AND VITAMIN A FROM HUMAN PLASMA.

Experiment 1.

Two 60 ml. samples of fresh human plasma were used. 40 ml. peroxide free ether has been added to each of them and mixed. One was kept at 25°C in a refrigerator for two hours, the other was kept for the same period of time at room temperature.

After re-thawing the frozen one, the ether layers of both were separated, and carotene and vitamin A were estimated. To the residues 60 ml. alcohol were added and two extractions with petroleum ether made, Carotene and vitamin A being again estimated.

Table 11..

	Frozen			Room temp.		
	EL.	R.	T.	EL.	R.	T.
carot.	13	28	41	3	33	36
vit.A	73	19	92	34	45	79

EL. Ether layer, R - residue, T. - total

Percentage of carotene and vitamin A in ether layers.

	Frozen	Room temp.
carot.	32	9
vit.A	79	43

Experiment 2.

The method was the same as in Experiment 1. of this series except that additional samples were used with petroleum ether and benzene as extracting solvents. The residues were shaken with equal volumes of the corresponding solvents and carotene and vitamin A were again estimated.

1. - organic solvent separated without shaking.

2. - second extraction, T. - total.

Table 12.

		Room temp. series. carotene,	vitamin A.	Frozen series. carotene,	vitamin A.
	1.	2	9	35	101
ethyl ether	2.	9	53	0	10
	T.	11	62	35	111
	1.	2	9	0	0
petr.ether	2.	0	10	0	19
	T.	2	19	0	19

		Room Temp. series.		Frozen series.	
		carotene,	vitamin A.	carotene,	vitamin A
	1.	0	10	0	0
benzene	2.	0	19	2	24
	T.	0	29	2	24

Experiment 3.

Freezing with liquid air.

50 ml. samples of fresh human plasma to which 25 ml. ether or petroleum ether has been added were used.

Some of the samples were frozen with the organic solvent, to other ether or petroleum ether have been added after freezing. All samples were gently stirred after addition of the solvent.

Freezing in liquid air was done by dipping a conical flask containing the mixture of plasma and organic solvent into the liquid air or ice and liquid air mixture for a minute or two till the mixture in flask was frozen hard.

Samples frozen in refrigerator were kept there for two hours.

After retawing the organic solvent layers were separated (1st extraction), carotene and vitamin A

estimated. Another extract was prepared by shaking the residues with 50 ml. of the corresponding solvent three times (2-ndextract). Carotene and vitamin A again estimated in the combined extracts.

To the residues of No. 1 and No. 5 fifty ml. To the residues of No. 1 and No. 5 fifty ml. alcohol was added and two extractions with 100 ml. petroleum ether made.

1. Frozen in liquid air then extracted with ether.....	±
2. Frozen in ice & liq. air....."....."..	±
3. Frozen in refrigerator " "	±
4. Left at room temperature " "	+
5. Frozen with ether in ice & liquid air...	+++
6. Frozen with ether in refrigerator.....	++++
7. Frozen in ice & liquid air then extracted with petrol. ether.....	-
8. Frozen with petroleum ether in ice & liquid air	-
9. Frozen with petroleum ether in refrigerator	-
10. Left ar room temperature, extracted with petrol. ether.....	-
11. Residue of No. 1.....	
12. Residue of No. 5.....	

Crosses indicate the intensity of the yellow colour of the first extracts. This colour as it has been mentioned includes some due to bilirubin (and possibly other pigments) which however is removed by the washing with potassium hydroxide. Hence the intensity of the colour of the crude extract affords no sure criterion of the amount of carotene extracted (see 1., 5., and 10).

Table 13

		Carotene	Vitamin A
	El.	0	0
1.	E2.	4	29
	T.	4	29
	El.	2	4
2.	E2.	5	43
	T.	7	47
	El.	0	0
3.	E2.	4	22
	T.	4	22
	El.	0	0
4.	E2.	2.	45
	T.	2	45
	El.	1	0
5.	E2.	9	36
	T.	10	36

		Carotene	Vitamin A
	El.	16	52
6	E2.	5	26
	T.	21	78
	El.	6	2
7	E2.	3	0
	T.	9	2
	El.	0	0
8	E2.	4	0
	T.	4	0
	El.	0	0
9	E2.	0	0
	T.	0	0
	El.	0	0
10.	E2.	5	0
	T.	5	0
Residue 1.		50	34
Residue 5.		42	34

Experiment 4.

Freezing with solid carbon dioxide.

To 50 ml. fresh plasma 20 ml. ether was added,

mixed and the mixture kept in a solid carbon dioxide chest for two hours. Another 50 ml. plasma mixed with 20 ml. ether was kept at room temperature for the same time.

After rethawing the ether layers were separated, carotene and vitamin A estimated.

Table 14.

	Frozen	Room temp.
carotene	27	4
vitamin A	61	40

Freezing undoubtedly causes an increase of the extractability of carotene and vitamin A from human plasma, but only when plasma is mixed previously with ethyl ether and frozen below - -25°C. About 50% carotene and 85% vitamin A goes to the ether layer.

Freezing plasma alone and then extracting has no effect on the extractability.

If instead of ethyl ether petroleum ether or benzene is used and the mixture frozen, no carotene or vitamin A goes to the petroleum ether or benzene layers.

From the experiment in which liquid air has been used for freezing it may be concluded that time plays an important role here. In that experiment plasma-ether mixture was kept in the liquid air only for a minute of two and though the temperature was much lower than - 25°C practically no carotene or vitamin A went into the ether layer. On the other hand a sample of the same plasma mixed with ether and kept for two hours in a refrigerator at - 25°C produced the normal effect, carotene and vitamin A in the ether layer. The effect of time may be exerted in a variety of ways.

It may be that time is required either for disruption of a carotene-protein complex (if such exists) or for the passage of carotene into solvent - or for both. It may be however that freezing below the eutectic point produces some denaturation of protein the amount of which is dependent upon time and that this in some way liberates the carotene to the solvent. These points receive further attention in later experiments (see page 76).

Fractional precipitation of plasma proteins.

Samples of 20 ml. of fresh citrated human plasma were used in this experiment. 50%, 75%, and 100% saturation with ammonium sulphate was obtained, then all samples were centrifuged and filtered under reduced pressure. The residues were washed with solution of ammonium sulphate of a corresponding concentration. The residues were then dissolved in 50 ml. of 0.9% sodium chloride solution, transferred to separating funnels and extracted the usual way with alcohol and petroleum ether. The precipitated proteins of 50% and 75% saturation dissolved completely in sodium chloride solution; in case of 100% saturation only partly.

Table 16.

	F.	55	87
50% saturation			
	Prpt.	--	--
	F.	--	--
75% saturation			
	Prpt.	65	83
	F.	--	--
100% saturation			
	Prpt.	50	83

The results of this and another similar experiment are given in tables below.

Table 15.

F - Filtrate, Prpt. - Precipitate

	Carotene	Vitamin A
	F. 18	145
50% saturation		
	Prpt. --	--
	F. --	--
75% saturation		
	Prpt. 18	150
	F. --	--
100% saturation		
	Prpt. 18	160

It is accepted that at 50% saturation with ammonium sulphate serum globulin is precipitated from plasma or serum. Neither carotene nor vitamin A was found with the precipitated proteins in the experiments described above, the total amount of both remaining with the unprecipitated protein which was mainly albumen. At 75% and 100% saturation all carotene and vitamin

A was found with the precipitated proteins.

The experiments would indicate that vitamin A and carotene accompany serum albumen as far as precipitation with ammonium sulphate is concerned.

Dialysis of plasma.

50 ml. of fresh human citrated plasma was dialysed against distilled water in a temperature of 0 C for 20 hours. Carotene and vitamin A were estimated in the dialysate and in the contents of the collodion sac.

As a control, two samples of the same plasma, 20 ml. each, were extracted twice with 40 ml. of petroleum ether after addition of 20 ml. of alcohol. Carotene and vitamin A were estimated in the combined extracts.

Results are given in the table below.

Table 17.

	Sac contents.	Dialysate.	Control.
Carotene	59	--	67
Vitamin A	133	--	150

Neither carotene nor vitamin A was found in the dialysate, which would indicate that both carotene and vitamin A exist in plasma in a form of not permeable particles either by itself or in a combination with other colloidal particles present in plasma.

The influence of bile salts on the extractability of carotene and vitamin A from human plasma.

50 ml. of fresh human citrated plasma was extracted twice with 100 ml. ethyl ether. Carotene and vitamin A were estimated in the combined extracts. Another 50 ml. of the same plasma to which 50 mgr. sodium taurogłycocholate dissolved in 5 ml. distilled water had been added, was extracted in a similar way. Carotene and vitamin A were again estimated. Yet another 50 ml. of the same plasma to which only 20 mgr. bile salts had been added were treated in the same way. Results are ġiven in the following table.

Table 18.

		Carotene	Vitamin A
Plasma alone	E.	20	92
	R.	42	20
	T.	62	112
Plasma with 40 mgr. bile salts added	E.	18	78
	R.	48	24
	T.	66	102
Plasma with 100 mgr. bile salts added	E.	10	74
	R.	56	32
	T.	66	106

Bile salts when added to plasma reduce the extractability ofcarotene and vitamin A with ether

quite distinctly if added in a comparatively large amount (100 mgr. per 100 ml. plasma). Only 50% of the carotene and 80% of the vitamin A could then be extracted. A smaller reduction in the extractability (carotene to 90% vitamin A to 85%) could be seen when the amount of bile salts added was only 40 mgr. per 100 ml. plasma.

It is not known whether this is only a physical phenomenon due to the lowering of surface tension or if bile salts are able to form a less extractable compound with carotene and vitamin A or carotene and vitamin A combined with other components of human plasma.

The inhibiting effect of bile salts is more marked in case of carotene than in the case of vitamin A.

Addition of a watery colloidal solution of carotene to human plasma before and after extraction with ether at different pH.

A colloidal solution of carotene in water was prepared by a method described in section I of this part. 5 ml. of the colloidal solution was made up to 25 ml. with water and the pH adjusted to 7.6. This was shaken three times with 50 ml. peroxide free ether, 10 minutes each time. All carotene was already extracted at the second extraction. The combined extracts were found to contain 125 γ carotene.

Five samples of fresh human citrated plasma, 25 ml. each were prepared as follows. The original pH of the plasma was 7.6.

1. pH 7.6 Extracted three times with 50 ml. peroxide free ether 10 minutes shaking each time. Carotene estimated in the combined extracts. To the residue 25 ml. 96% alcohol was added and three extractions with 50 ml. petroleum ether made, carotene estimated in the combined extracts.

2. pH 7.6 — Extracted as above, then 5 ml. of the colloidal carotene (125 γ) added, left for some time then again three extractions with 50 ml. ether made. The residue was worked up as in No. 1.

3. pH adjusted to 6.7 — Extracted as No. 1. pH restored to 7.6, then 5 ml. of the colloidal carotene added and after a few minutes another three extractions with ether made. The residue was treated as in No. 1.

4. pH adjusted to 5.6 — Extracted as No. 1. pH restored to 7.6 then 5 ml. colloidal carotene added and after a few minutes three extractions with ether made. The residue was treated as in No. 1.

5. pH 7.6 — No extractions made before the addition of 5 ml. of colloidal carotene. Three extractions with ether made. The residue was worked up as in No.1.

Results of this experiment and of a similar one are given in the tables below (expressed as usual in γ of carotene per 100 ml. plasma or colloidal

solution of carotene).

Table 19.

	First series of extractions	Second series of extractions	Residue	Totals
C.	508	--	--	508
1.	40	--	52	92
2.	40	412	152	604
3.	72	520	12	604
4.	88	476	6	570
5.	--	136	460	596

Table 20.

	First series of extractions	Second series of extractions	Residue	Totals
C.	96	--	72	168
1.	24	--	48	72
2.	24	76	140	240
3.	28	84	108	220
4.	48	180	4	232
5.	--	40	232	272

In the second experiment the original pH of plasma was 8.1. No. 3 was adjusted to pH 6.7, No. 4 to pH 5.7. All other operations as in the first experiment. C. in both experiments stands for colloidal solution of carotene.

Conclusions.

When 500 γ carotene is added to 100 ml. plasma in form of a watery colloidal solution it is found that even after repeated shaking with ether about 450 γ will be retained in the 100 ml. plasma. Recently Hoch (21) examining the effect of prolonged administration of carotene in form of vegetables on the level of carotene and vitamin A in blood, found that the maximum amount of carotene held by 100 ml. plasma was 386 γ in one case and 551 γ in another.

If however plasma be first extracted with ether and then an attempt made to introduce colloidal carotene it is found that only about 20% of that previously held is now retained. If the preliminary extraction with ether be carried out at pH slightly below 7, hardly any carotene will be now held. The same applies if the preliminary extraction is carried out at pH 5.6. At this pH however a marked precipitation of plasma protein occurs. From the previous experiments we know already that by simple extraction of plasma with ether only a small amount of carotene and about 50% vitamin A can be extracted. When however the pH is shifted slightly below 7, a jump in the

extractability of both is seen. On the other hand the ability of plasma for holding carotene decreases after it has been extracted with ether and practically disappears if the preliminary extraction with ether be carried out at a pH just below 7.

Keeping in mind these two facts one could suppose that by shaking plasma with ether, other substances (cholesterol, lipoids) beside carotene and vitamin A are being extracted, which normally help carotene and vitamin A to be held in plasma, or that by the named operation a disruption of certain bonds occurs which bind carotene to a plasma protein.

Investigation of ether extracts of plasma made at different pH for sunstances accompanying carotene and vitamin A (cholesterol and lipoids).

Four samples of 50 ml. of fresh citrated human plasma were taken.

1. left at original ,pH i.e.7.6
2. adjusted to pH 6.5
3. " " pH 5.4
4. re-adjusted to pH 7.6 from pH 5.4.

All samples were extracted twice with 100 mls. peroxide free ether, ten minutes each time. Ether was distilled off from the combined extracts, the residue dissolved in petroleum ether, washed with 3% KOH and water, distilled down to 50 ml, divided in two 25 ml. portions, one taken for estimation of carotene vitamin A and cholesterol, the other for estimation of lipoid phosphorus. To the residues 50 ml. alcohol was added, two extractions with petroleum ether made and the extracts examined as above. The table below gives the results. All figures are given for 100 ml. plasma, Carotene and vitamin A are expressed in γ and I.U., cholesterol and lipoid phosphorus in mgr.

Table 21.

		Carotene	Vitamin A	Cholesterol	Lipoid P.
	E.	14	112	38	0.3
1.	R.	60	6	122	5.8
	T.	74	118	160	6.1
	E.	26	138	46	0.4
2.	R.	52	2	101	5.7
	T.	78	140	147	6.1
	E.	36	120	87	0.6
3.	R.	44	14	80	5.48
	T.	80	134	160	6.08

		Carotene	Vitamin A	Cholesterol	Lipoid P
	E.	22	108	52	0.4
4.	R.	48	12	101	5.6
	T.	70	120	153	6.0

In the next table are given the amounts of carotene, vitamin A, cholerterol and lipoid phosphorus in extracts expressed as percentages of totals.

Table 22.

	pH 7.6	pH 6.5	pH 5.4	pH 7.6 - 5.4
Carotene	19	33	47	31
Vitamin A	87	99	91	90
Cholesterol	24	31	56	34
Lipoid P.	5	7	10	6.6

Next table gives the results of a similar experiment in which the pH values were as follows:

1. pH 7.65
2. pH 6.8
3. pH 5.2
4. pH 7.65 from 5.2

Table 23.

		Carotene	Vitamin A	Cholesterol	Lipoid P.
	E.	14	60	43	0.4
1.	R.	56	40	92	6.0
	T.	70	100	135	6.4

		Carotene	Vitamin A	Cholesterol	Lipoid P.
	E.	20	84	53	0.68
2.	R.	44	28	85	5.36
	T.	64	110	138	6.04
	E.	44	98	115	0.9
3.	R.	20	16	30	5.44
	T.	64	114	145	6.34
	E.	28	76	65	0.56
4.	R.	36	32	72	5.56
	T.	64	108	137	6.12

The percentages of the substances under examination in extracts are given in the following table.

Table 24.

	pH 7.65	pH 6.8	pH 5.2	pH 7.65-5.2
Carotene	21	30	67	43
Vitamin A	55	77	91	70
Cholesterol	31	38	83	46
Lipoid P.	6.5	11	14	9

Both experiments gave similar results, showing that with a decrease in the pH of plasma there is an increase in the extractability of not only carotene and vitamin A but also of cholesterol

and lipoids. There seems to be a rough parallelism especially in the extractability of carotene, vitamin A and cholesterol. The figures for lipoid phosphorus, though the absolute differences were in both cases small, agree with the view that ether extractability of phospholipins is also a function of pH.

DENATURATION OF PLASMA PROTEIN BY SHAKING WITH ETHER.

In this and in the following experiments 25 ml. samples of fresh human citrated plasma were used, the pH being adjusted to different values. All samples were shaken by hand with 50 ml. ether in separating funnel twice for 15 minutes. The extracted samples were filtered and then exposed to diminished pressure for three hours to get rid of ether. The precipitated protein was separated by filtering. Two ml. of the filtrate were taken for the estimation of total nitrogen and another five for estimation of N.P.N.

The pH values of the first experiment were as follows:

1. pH 10
2. " 8 (original)
3. " 6.8
4. " 5.5
5. " 4.7

In one sample at the original pH total nitrogen and N.P.N. were estimated without shaking with ether (control).

Results of the first experiment are given in the table below. Figures given for 100 ml. plasma in all experiments.

Table 25.

pH	10.0	8.0	6.8	5.5	4.7	Control
Gms.protein in filtrate	5.08	5.93	5.29	4.98	4.76	6.07
protein in filtrate as % of that originally present	83.7	97.9	87.3	82. 0	78.5	1000
% protein denatured and precipitated	(16.3)	2.1	12.7	18.0	21.5	--

In the second experiment the pH of all samples after extraction were re-adjusted to 4.7 then filtered, exposed to diminished pressure and further treated as in first experiment.

Table 26.

pH	12.2	9.6	7.7	6.5	5.5	4.7	Control
Gms.protein in filtrate	0.48	4.89	5.27	5.22	5.12	4.74	5.36
protein in filtrate as % of that originally present.	0.9	91.2	98.3	97.4	95.5	88.4	100.0
% protein denatured plus denatured and precipitated	99.1	8.8	1.7	2.6	4.5	11.6	--

The table below gives the results of another experiment of the same type in which, after shaking with ether, filtering and getting rid of ether from the filtrates (filtrate 1.) protein was estimated and then after re-adjusting pH to 4.7 and filtering (filtrate 2.) protein was again estimated. By the first filtering protein denatured and precipitated was separated while by the second filtering protein denatured but precipated only after bringing pH to 4,7 was removed.

Table 27.

pH	12.0	10.1	7.9	6.8	5.4	4.7	Control
Gms.protein in filtrate 1.	5.0	5.0	5.24	4.84	4.63	4.41	5.31
Protein in filtrate 1 as % of that originally present.	94.0	94.0	98.6	91.0	87.2	83.0	100.0
% protein denatured and precipitated	(6.0)	(6.0)	1.4	9.0	12.8	17.0	--
gm.protein in filtrate 2.	0.58	4.63	5.04	4.84	4.63	4.51	--
% protein in filtrate 2.	10.8	87.2	95.0	91.0	87.2	84.9	--
% protein denatured, unprecipitated	89.2	12.8	5.0	9.0	12.8	15.1	

Table 28 gives the results of yet one experiments which was performed in exactly the same way as the previous one.

Table 28.

pH	12.1	10.0	7.3	6.6	5.4	4.7	Control
Gm. protein in filtrate 1.	5.23	5.6	5.49	5.38	5.22	4.95	5.60
protein in filtrate 1 as % of that originally present	93.4	100.0	98.0	95.8	93.0	88.4	100.0
% protein denatured and precipitated	(6.6)	0.0	2.0	4.2	7.0	11.6	--
gm. protein in filtrate 2.	0.032	5.27	5.42	5.42	4.95	4.73	
%protein in filtrate 2.	0.6	94.0	97.0	97.0	88.4	84.8	--
% protein denatured, unprecipitated	99.4	6.0	3.0	3.0	11.6	15.6	--

It is seen from these experiments that by shaking plasma with ether at different pH protein of plasma is precipitated to different degrees. With movement to the acid side it increases from about 2% at pH 7.7 to 17 % at pH 4.7 after half an hour shaking with ether. The increasing amount of precipitated protein may be observed even by eye. Movement to the alkaline side of the pH range does not produce any visible precipitation of

protein but increases the viscosity of plasma making filtering more difficult and giving a less viscous filtrate containing less protein. During shaking of plasma with ether some protein denatures without precipitation which, however, precipitates after the pH is re-adjusted to pH 4.7.

A controlling experiment in which pH of samples were adjusted similarly as in previous experiments but no shaking with ether done failed to produce enough evidence that the denaturation and precipitation of plasma protein is a function of pH only, through there was a slight precipitation of protein at pH 4.7.

General results of the experiments on denaturation of plasma protein by shaking with ether are given in the following table.

Table 29.

	pH 7.7	pH 6.7	pH 5.5	pH 4.7
% protein denatured and precipitated	2.0	8.4	12.3	17.0

DENATURATION OF PLASMA BY FREEZING WITH ETHER.

Two samples of fresh human citrated plasma 25 ml. each were mixed with 20 ml. ethyl ether. One wasleft at room temperature the other was kept in solid carbon dioxide for 4 hours. After re-thawing and separating of the ether layers both samples were filtered and then exposed to reduced pressure for three hours to get rid of ether dissolved in them. Protein was estimated in both cases (filtrate 1.) Then to 5 ml. of both of the filtrates Magnezium sulphate was added to produce saturation and after filtering, protein was again estimated in the filtrates (filtrate 2.) Table 30 gives the results per 100 ml. plasma.

Table 30.

	Control	Frozen
Gm. total protein in filtrate 1.	5.31	5.04
protein in filtrate 1 as % of that originally present	100.0	95.0
% protein denatured and precipitated	0.0	5.0
gm. serum albumen in filtrate 2.	2.72	2.5
serum albumen in filtrate 2 as % of that originally present.	100.0	92.0

	Control	Frozen
% of serum albumen denatured and precipitated	0.0	8.0

It is seen from this experiment that the protein denatured and precipitated from plasma by freezing with ether can be accounted for as serum albumen.

Table 31 gives the results of a similar experiment in which however the loss of serum albumen was not investigated.

Table 31.

	Control	Frozen
Gm. protein in filtrate	5.6	5.27
protein in filtrate as % of that originally present	100.0	94.0
% protein denatured and precipitated	--	6.0

The results of both experiments indicate that by freezing plasma at original pH with ether in solid carbon dioxide about 5% of plasma protein is denatured and precipitated. The results of the first experiment provide evidence that the protein in question is serum albumen.

EXPERIMENTS ON COLLOIDAL WATERY SOLUTIONS OF CAROTENE.

PREPARATION OF A COLLOIDAL SOLUTION OF CAROTENE.

This was done according to the method usually applied (H.v. d. Bergh, Drummond, With etc.) by mixing an acetone solution of carotene with water and then evaporating the acetone under reduced pressure.

A solution of carotene thus obtained may have colour from yellow to deep orange depending on the amount of carotene used.

The colour of such solution disappears gradually if the solution is left standing . This is due to oxidation of carotene. In darkness the process of oxidation goes slower.

The dispersion of carotene in the water phase depends,probably, on the relative amounts of carotene, acetone and water used.

Some of the properties of such colloidal solutions depend probably on the degree of dispersion of carotene. For example, from some solutions obtained in the above way carotene was extracted with ether by a single extraction,while from other at the same pH it could not be extracted even after three prolonged extractions.

In certain of the Experiments already described colloidal solutions of β-carotene prepared in this way were used.

INFLUENCE OF pH ON THE EXTRACTABILITY OF CAROTENE FROM COLLOIDAL AQUEOUS SOLUTIONS, WITH ETHER AND PETROLEUM ETHER.

In this experiment a colloidal aqueous solution of carotene containing 92 γ in 15 mls. was used.

Six pairs of 15 mls. samples were prepared each pair being adjusted to different pH. One sample of each pair was extracted with ethyl ether the other with petroleum ether, five minutes shaking being applied.

Table 32.

		Ethyl ether extr.		Petrol. ether ext.	
1	pH 2.5	92	100%	48	52%
2	pH 3.52	48	52%	38	41%
3	" 5.62	48	52%	2	2%
4	" 6.04	36	39%	4	4%
5	" 6.5	32	35%	5	5%
6	" 7.13	22	24%	4	4%

At pH 2.5 the extraction by ethyl ether is complete no carotene being left in water solution. When pH increases the amount of carotene extracted decreases, already at pH 3.52 being less than 50%. With further increase of pH the extractability still decreases the majority of carotene remaining in water. The amount of carotene extracted by petroleum ether is much smaller than by ethyl ether.

INFLUENCE OF pH ON THE EXTRACTABILITY OF CAROTENE FROM COLLOIDAL AQUEOUS SOLUTIONS AND FROM 1% SERUM ALBUMEN SOLUTIONS BY ETHYL ETHER AND BY PETROLEUM ETHER.

Samples of 18 mls. water or albumen solutions containing 93γ per sample were used. 20 mls. ether or petroleum ether was used for extraction, five minutes shaking being applied.

The pH were adjusted as follows:

	Water systems	Albumen systems
1	pH 3.0	1 pH 2.32
2	" 6.3,(original)	2 " 2.85
3	" 7.0	3 " 7.6 (original)
4	" 8.7	4 " 8.4

Table 33.

Water systems.

			Ethyl ether extr.		Petrol.ether extr.	
1	pH	3.0	93	100%	63	66%
2	"	6.3	85	91%	10	11%
3	"	7.0	48	52%	5	6%
4	"	8.7	45	48%	9	10%

Serum albumen systems.

1	"	2.32	16	17%	Separation of petr oleum ether layers has not been possible as a jelly was formed throughout the systems.
2	"	2.85	11	12%	
3	"	7.65	13	14%	
4	"	8.7	11	12%	

THE INFLUENCE OF ETHYL ALCOHOL ON THE EXTRACTABILITY OF CAROTENE FROM ITS COLLOIDAL AQUEOUS SOLUTIONS AT PH 7.4 BY ETHER.

A colloidal solution of carotene in water containing 122γ per 15 mls. was prepared by the usual method.

Five samples 15 mls. each were used. Following increasing amounts of ethyl alcohol were added.

1. 0.5 ml.
2. 1.0 ml.
3. 5.0 mls.
4. 10.0 mls.
5. 15.0 mls.

Another five 15 mls. samples of colloidal carotene in 1% serum albumen were prepared, again 0.5, 1.0, 5, 10, and 15 mls. ethanol added. All systems were extracted by 30 mls. ether, 5 minutes shaking being applied.

Table 34.

(water systems)

Control, no alcohol added---------122γ extracted - 100%

1.	0.5 ml. alcohol added	(3.3%)	113	extracted	-93%
2.	1.0 " " "	(6.2%)	100	"	82%
3.	5.0 " " "	(25%)	122	"	100
4.	10 mls. " "	(40%)	120	"	100%
5.	15 " " "	(50%)	118	"	100%

Extraction was complete in all cases.

1 % albumen systems

Control	no alcohol added	12γ Extracted	--------	10%
1	0.5 ml. alcohol added	8γ	" ----------	7%
2	1.0 " " "	8γ	" ----------	7%
3.	5.0 " " "	15γ	"	12%
4.	10.0 " " "	120γ	"	100%
5.	15.0 " " "	122γ	"	100%

In case of watery solutions carotene was extracted totaly already without addition of any alcohol. Addition of alcohol did not increase the extractability.

In the case of albumen solutions the influence of ethanol is more marked. It is negligible up to 25% of alcohol but bringing a complete extractability when 40 and 50 % alcohol was attained.

INFLUENCE OF ETHANOL AND AMMONIUM SULPHATE ONTHE EXTRACTABILITY OF CAROTENE FROM AQUEOUS COLLOIDAL SOLUTIONS AND FROM SOLUTIONS OF 1% SERUM ALBUMEN BY ETHER AT pH 2.5 and 7.3

An acqueous solution of carotene and a solution of carotene in 1% serum albumen, both containing 125γ per 15 mls. were used. Four groups of systems were prepared. Two groups contained water systems, the other two albumen systems. One group of the water systems had pH 2.5, the other one pH 7.3. The same were the pH of the albumen systems. Each group had three systems. First was a control, 15 mls. watery or albumen solution of carotene alone. To second 5 mls. alcohol has been added, to the third ammonium sulphate to saturation. The groups are listed below.

	water systems		albumen systems	
	1. control		1. control	
pH 2.5	2. 5 mls. ethanol	pH 2.5	2. 5 mls. ethanol	
	3. amm. sulphate		3 amm. sulphate	
	1. control c		1. control	
pH 7.3	2. 5 mls. ethanol	pH 7.3	2.5 mls. ethanol	
	3 amm. sulphate		3 amm. sulphate	

All systems were shaken with 25 mls. ethyl ether during five minutes.

Results given in γ of carotene and in % of extracted carotene.

Table 35.

Water systems

	1.	125	100 %
pH 2.5	2.	125	100 %
	3.	125	100 %
	1.	11	9 %
pH 7.3	2.	81	65 %
	3.	95	76 %

albumen systems.

	1.	10	8 %
pH 2.5	2.	12	10 %
	3.	125	100 %
	1.	9	7 %
pH 7.3	2.	18	14 %
	3.	74	59 %

At pH 7.3 in the aqueous system only 9 % carotene was extracted but after addition of 5 mls. alcohol (to attain 25 % ethanol solution) the extractability increased to 65 % while ammonium sulphate brought it up to 76 %. At pH 2.5 carotene was extracted completely without addition neither alcohol nor ammonium sulphate.

In case of albumen systems the alcohol had practically no influence. This in concordance with a previous experiment in which also alcohol in 25 % strength had no influence on extraction of carotene from albumen solutions, 40 or 50 % being required to bring complete extraction at pH 7.4. Ammonium sulphate on the other hand has a marked effect in this case giving 60% extraction at pH 7.3 and 100 % extraction at pH 2.5.

The inhibiting effect of sodium taurglycocholate on the extractability of carotene from colloidal watery solutions.

A colloidal solution of carotene in water, containing 275γ of carotene per 100 mls. has been prepared. This solution was shaken three times with 200 mls. ether each time 10 minutes. 225γ were extracted and 50γ left in the water as residue. Another 100 mls. of the same colloidal solution, to which 100 mgr. of sodium tauroglycocholate has been added, was shaken in the same way as above. This time only 12.5 γ have been extracted the rest remaining in the water solution.

The experiment shows that the inhibiting effect of bile salts on the extractability of carotene from its watery colloidal solution is striking and much greater than that on the extractability of the same substance from human blood plasma. (Table 18).

When 100 mgrs. of bile salts has been added to 100 mls. of plasma, the extractability of carotene has been reduced to 50% of that without addition of bile salts, while addition of the same amount of bile salts to 100 mls. colloidal solution of carotene reduced the extractability to 4.5% of that without addition of bile salts, 95.5 % remaining in water solution.

In this case, as in other, a quantative difference in the behaviour of plasma carotene and colloidal watery solutions of carotene may be observed.

The effect of freezing on a colloidal aqueous carotene solution.

Two samples of 50 mls. of colloidal carotene were used. To each of them 20 mls. ether was added. One was left at room temperature, the other kept in refrigerator at - 24°C for two hours. After re-thawing the ether layers were separated and carotene estimated.

Table 36.

	Frozen	Room temp.
Carotene	107	34

Conclusion.

Freezing causes that a greater amount of carotene

passes into ether layer as compared with a similar system left at room temp. A similar effect has been recorded when plasma was frozen with ether below - 25°C.

Freezing of a 1 % solution of serum albumen containing colloidal carotene showed to be without effect in one experiment which was performed.

ADSORPTION OF VITAMIN A ON DRIED SERUM ALBUMEN.

0.5, 1, and 2 gms. samples of serum albumen were weighed on watch glasses. The albumen powder was covered with a solution of vitamin A concentrate in petroleum ether. After evaporation of petroleum ether the albumen was dissolved in water and filtered after half an hour. To 50 mls. of the filtrate 50 mls. alcohol was added and an extraction with 100 mls. petroleum ether made. Vitamin A estimated in the extracts.

Following systems were prepared.

1. 1 gm. albumen dissolved in 200 mls. water (no vitamin added)
2. 0.5 gm. " " " 100 mls. water
3. 0.5 gm. " " " 200 " "
4. 1 gm. " " " 200 " "
5. 2 gm. " " " 400 " "

Results in I.U. vitamin A.

Table 37.

	In 50 mls. filtrate.	In total filtrate.	Per 1 gm. albm.
1.	5.8	23.2	23.2
2.	67.4	134.8	269.6
3.	46.5	186.0	372.0
4.	81.3	325.0	325.0
5.	81.3	650.0	325.0

Vitamin A concentrate which alone is insoluble in water passes into watery solution in contact with serum albumen. The nature of the combination which may be simple adsorption, is unknown. It must be remembered that the substance used was not pure crystalline vitamin A but a concentrate containing probably some oil of unknown origin. The substance was obtained from B.D.H. under the name of vitamin A concentrate.

DISCUSSION AND RESULTS

The aim of the experiments described in the foregoing part was to gather as many as possible facts about the behaviour of carotene and vitamin A in plasma and from these facts to deduce, if possible, the form in which these two substances exist in that medium. For the sake of comparison more simple systems than plasma were also examined. These were: colloidal solutions of carotene in water and in 1 % serum albumen solution.

One of the characteristics of blood is its stability of pH. Small deviations from the normal pH of blood cause serious changes and endanger the life of the organism. Investigation then of the changes accompanying a marked change in the pH of human blood in vivo is impossible. However the pH of plasma withdrawn from the body may be changed freely in vitro and the accompanying phenomena observed. Thus if fresh human citrated plasma, whose pH is in the region of 7.6, be shaken with ether vigorously for half an hour no visible change occurs except for a slight passage of a yellow pigment to the ether layer and sometimes a slight but perceptible precipitation of plasma protein. When however the pH is moved slightly below 7, say to 6.8, and shaking with ether is applied, much more passage of the yellow pigment to ether layer

occurs and is accompanied by an increased precipitation of protein. With further movement to the acid side the amount of the pigment and the rate of its passage increases still further, being greatest at a pH value between 4 and 5. The amount and the rate of precipitation of plasma protein increase similarly, being greatest in the region of pH 4.7, which is the isoelectric point of serum albumen. Still further movement to the acid side causes a decrease in the passage of the pigment to the ether layer and partial re-dissolving of the precipitated protein occurs. Difficulties arise at pH about 3 and lower when a jellied mass is formed throughout the system and separation of the ether layer becomes impossible. It is however seen within the practical limits of the experiment that lowering of pH of plasma from the original value to about pH 4 and shaking with ether produces an increase in the pigmentation of the ether layer accompanied by an increased precipitation of plasma protein. Movement to the alkaline side does not cause any marked increase in the pigmentation of the ether layer nor does any precipitation of plasma protein occur. Examination of the ether extracts of plasma proved the presence of carotene, vitamin A, bilirubin, cholesterol and lipoids.

Many workers have pointed out that while it

was impossible to extract carotene from human plasma by simple shaking with ether, the whole of the pigment could be extracted by addition of a certain volume of alcohol or other protein precipitating agents. It appears that what those workers caused by addition of alcohol, i.e. total precipitation of plasma protein, may be obtained gradually by changing the pH of plasma to the acid side and shaking with ether. The increased pigmentation of the ether layer and the correspondingly increased precipitation of the protein may be seen even by eye. On the other hand the residue become paler as the pH is moved to the acid side, being colourless at pH between 4 and 5, when the whole of the pigment has been yielded to the ether layer.

It may then be concluded that the extraction of carotene and vitamin A from human plasma is a function of pH. But is it a function of pH only i.e. can carotene and vitamin A be extracted totally from plasma by using other means at a pH at which it could not be extracted by shaking with ether only? The answer is in the affirmative because addition of alcohol or other protein precipitating agents renders carotene and vitamin A extractable with ether or petroleum ether. In this case the effect of pH was eliminated, the only factor, influencing extraction being alcohol, which

is known to cause denaturation and precipitation of plasma protein. It is thus to be noted that those circumstances which favour the extractability of carotene and vitamin A involve denaturation and /or precipitation of the proteins. On the other hand, precipitation without denaturation (as by ammonium sulphate at pH 7.6) does not render the carotene ether soluble. The question arising now is whether denaturation and precipitation of plasma protein is necessary to make carotene and vitamin A extractable. If this be the case it would mean that some sort of bond exists between a plasma protein on one hand and carotene and vitamin A on the other hand, and that the bonds are annihilated by the denaturation action of alcohol and other agents. The next question, if the first is to be given an affirmative answer is whether denaturation alone is sufficient for the liberation of carotene and vitamin A or if it must be accompanied by precipitation. Both these questions find their answer in the results of the experiment on denaturation of plasma protein by shaking with ether (Table 28). It is seen from these results that denaturation alone does not liberate carotene and vitamin A to the solvent. At pH 12 denaturation of plasma protein is almost complete but no precipitation occurs, and no increase in the extractability

of carotene and vitamin A.as compared with that at original pH is noticed. On the other hand where precipitation of the denatured protein occurs a marked extraction of carotene and vitamin A is seen (pH 6.8 to 4.7). That the precipitated protein is denatured may be seen from the fact that it is soluble in neither water nor in normal saline.

The kind of denaturation involved here is most probably surface denaturation caused by shaking and increased by the presence of ether. Ether itself according to W.D. Bancroft and T. Rutzler (22) does not denature protein (egg albumen). The so-called denaturation caused sometimes by ether is a result of removing water adsorbed on the protein particle. Shaking, on the other hand does cause surface denaturation. Bull and Neurath (23) studied surface denaturation of egg albumen by shaking and found it to be dependent on the time of shaking and on the pH of the albumen solution, the rate of denaturation being greatest at the isoelectric point of the protein. It is mostprobable that denaturation by shaking is increased by the presence of ether. According to more recent investigations by W. T. Astbury and R. Lomax (24) denaturation, among other changes, is accompanied by unfolding of the globular structure of the peptide chains to a more

linear one, and when precipitation occurs the peptide chains acquire a parallel structure. Simultaneously certain new groups appear (and probably other disappear). This explanation may to some extent account for the disappearance of the bonds holding carotene, vitamin A and bilbirubin on a plasma protein, thus setting them free and rendering them extractable. Denaturation then and precipitation of plasma protein is a condition which must be fulfilled before carotene and vitamin A can be extracted from human plasma. This of course involves the acceptance of the view that these two substances are bound with a plasma protein in form of a complex. What sort of combination it is cannot be answered at this stage of the research. The bonds binding carotene and vitamin A with the protein are probably of a kind similar to those by which prosthetic groups are bound in conjugated proteins, resembling the way in which reduced heme is combined with native globin. C.L.A. Schmidt (25) says:-

> "The compound of reduced heme and native globin in neutral solutions is a "firm"

> compound. The compound of reduced heme and denatured in neutral solutions is a "loose" compound..... In acid solution there is both denaturation of the protein and loosening of its bond with heme. Whether in this case the loosening of the bond is due to the acid or to the denaturation is not known. The indications are that in all conjugated protein of the hemoglobin type in which the prosthetic group is firmly bound to the protein, the prosthetic group can be separated from the protein only when the protein is denatured."

The results of the experiments on denaturation of plasma protein by shaking with ether at different pH described in this work suggests an extension of Schmidt's view to the case of carotene and vitamin A in plasma. One modification however must be made, namely that in the case of carotene and vitamin A precipitation of the denatured protein is necessary for disruption of the bonds binding these substances to the protein.

Further evidence to support the view that the disruption of the carotene-protein-vitamin A complex by shaking with ether is caused by

denaturation and precipitation of the protein is provided by the reduced ability for holding carotene possessed by plasma whose protein has been partially denatured and precipitated by shaking with ether. Thus 100 ml. fresh citrated plasma containing about 100γ carotene could at pH 7.6 (original) bind another 400γ introduced as aqeous colloidal solution, which could not be extracted even after repeated shaking with ether. On the other hand 100 ml. of the same plasma previously extracted with ether at pH 7.6 was able to bind only 20 % of that amount. When however the same plasma was extracted first at pH 6.8 or 5.4 and the pH was then re-adjusted to 7.6 practically no carotene could be introduced in a non-extractable form by addition of colloidal carotene solution. The explanation is that in the first case (plasma un-extracted) the plasma protein was native, thus possessing intact all bonds for binding carotene. In the second case (plasma extracted previously with ether at pH 7.6) by shaking with ether part of the protein was denatured and precipitated and a part of the bonds lost. By extraction at pH 6.8 and 5.4 still more protein was denatured and precipitated, most of the bonds being destroyed, so that added carotene remained free

and could be almost totally extracted.

Investigation of the ether and petroleum ether extracts of human plasma showed that carotene and vitamin A are extracted not only together with bilirubin but also with cholesterol (cholesterol esters) and lipoids. The amount of cholesterol and.lipoids extracted was also proved to be function of the denaturation and precipitation of plasma protein, increasing with the lowering of pH of plasma and shaking with ether. This fact of course suggests that not only carotene, vitamin A and bilirubin but also cholesterol (cholesterol esters) and lipoids may be in some way bound to a plasma protein from which they may be liberated only by denaturation and precipitation of the protein. The percentage of lipoids, though increasing with the increase of the other substances, is several times smaller. The small extractability of lipoids may be explained by the assumption that lipoids are more built into the structure of the protein while the other bound to the surface of the protein particle. Denaturation then of the surface has therefore a greater effect on carotene, vitamin A, bilirubin and cholesterol than on lipoids.

There are indications that pH itself excercises some influence on the protein-carotene-vitamin A complex. This may be seen from the results of the experiments in which pH of plasma was lowered from the original value 7.6 to pH 5.4 and brought back to the original pH. The amount of carotene and vitamin A extracted after this operation was greater than at the original pH but smaller than at pH 5.4 This indicates that by lowering of pH some loosening of the bonds between protein on the one side and carotene and vitamin on the other side occurs, which may be partly restored by re-adjusting of the pH to the original value. Similar changes in which a loosening of the bonds binding a prosthetic group to a protein occurs by changing of pH are well known in the chemistry of enzymes. An example may be the Warburg-Christian flavo-protein which in acid solution is reversibly resolved into protein and lactoflavin phosphate and which re-combines in neutral solution to the original flavoprotein (26).

Having concluded that there do exist in human plasma carotene-protein and vitamin A--protein complexes, the next question to answer is, which is the protein that participates in

the complexes.* This question is more difficult to answer without cataphoretic experiments being performed but certain facts will be given presently which agree with the view that the protein in question probably is serum albumen in both cases.

It has been already mentioned that carotene and vitamin A in plasma extracts were always accompanied by bilirubin which was then washed out with 3% potassium hydroxide. The amount of bilirubin was proportional to the amount of carotene and vitamin A, increasing as the pH was moved to the acid side and being complete at pH 4.7. This parallelizm between carotene and vitamin A on the one hand and bilirubin on the other strongly suggests that bilirubin is bound like carotene and vitamin A to a plasma protein, being liberated by the same factors i.e. denaturation and precipitation of the protein.

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* It is of course possible that there may be a single carotene-protein-vitamin A complex, i.e. that molecules of both vitamin and pro vitamin may be bound by a single protein molecule. Alternatively different molecules of the same protein may be combined with carotene and vitamin-A respectively, and a third possibility is that these two substances may be attached to different proteins.

K. O. Padersen and J. Waldenström (27) have proved by studies employing electrophoresis and the ultracentriguge that both kinds of bilirubin present in plasma i.e. that giving direct (immediate direct) and that giving indirect (delayed direct) V. d. Bergh reaction, are bound to serum albumen. They further proved this by showing that bilirubin added to a solution of serum albumen is immediately bound to it while in a solution of egg albumen it remains free. The fact then than carotene, vitamin A and bilirubin are liberated simultaneously and proportionately by the same factor strongly supports the view that all these substances are bound to the same protein, (serum albumen). The results of the experiments on fractional precipitation of plasma protein agree with this view. No carotene or vitamin A could be found with the globulins precipitated by 50% saturation with ammonium sulphate, but all carotene and vitamin A were recovered with the serum albumen fraction.

Contradictory results, namely that carotene is bound in plasma to serum globulin and only traces to serum albumin, were obtained by H. Bennhold (28) by cataphoretic studies. This author strongly criticises the

precipitation methods regarding cataphresis as the best method to be applied for studying plasma proteins. It seems however that neither method is too perfect and that a great possibility exists that the application of a strong electric field may produce changes in the bonds binding carotene to protein as much or even more as introduction of ammonium sulphate.

McFarlane (29) found that lipoid fraction of human serum, which cannot normally be extracted with ether, passes readily to this solvent when serum is mixed with ether and frozen below -25°C. He explains this is due to disruption of a protein-lipoid complex normally existing in serum. The results of the freezing experiments described in this work show that by freezing human plasma with ether carotene and vitamin A also pass to the ether layer without shaking (other than the initial mixing) being applied. The amount of carotene is 4 to 7 time greater than that which goes to the ether layer at room temperature, while the amount of vitamin A is roughly doubled. Taking into consideration McFarlane's

view about the protein-lipoid complex and the carotene-protein-vitamin A complex, and the fact carotene and vitamin A are liberated simultaneously with lipoids by the same cause (freezing with ether) it may be supposed that there is actually in plasma or serum one complex of carotene, vitamin A, lipoids, bilirubin and a protein, (serum albumen) or at least that serum albumen forms complexes with all of them. Bilirubin has been found to accompany carotene and vitamin A in the liberation by freezing as well. The disruption of the complex by freezing is again most probably caused by partial denaturation of the protein of the complex. The loss of the denatured protein as Table 30 shows could be accounted for in the serum albumen fraction. This again supports the results obtained by fractional precipitation of plasma protein and the reasoning on the results of Paderson and Walderstrom from which was concluded that the protein on which not only bilirubin but also carotene and vitamin A are bound in plasma or serum is serum albumen.

The experiments on aqueous colloidal solutions of carotene and on carotene in 1 % albumen solution were performed because of the light expressed by V. d. Bergh and Muller (5) that simple colloidal aqueous solutions of

carotene behave similarly to plasma carotene. The results though in some respects resembling those obtained from the experiments on plasma and serum, do not contradict the statements expressed in the present discussion. First of all the properties of watery colloidal solutions of carotene are not always the same. In some cases carotene may be extracted from such solution totally with ether at pH 7.6 (a fact never observed in case of plasma carotene) and in others even after repeated shaking with ether at the same pH part of the carotene remains in the water phase. In such cases pH exerts its influence but to an extent different than observed in the case of plasma carotene. Thus at a pH between 5 and 6, 48 % carotene was extracted from aqueous carotene solution while from plasma at the same pH 70 % could be extracted. At a pH between 3 and 4 again 48 % was extractable from aqueous solution while from plasma 86 % carotene was extracted at the same pH. In watery colloidal solutions of carotene acidity probably produces a change in the dispersion of the colloidal particles, rendering them more extractable with ether. It may be seen even by eye that at a very low pH

the colloidal particles tend to sedement. A similar difference is seen in case of 1 % serum albumen solution of carotene and plasma. At pH about 3, 86 % carotene may be extracted from plasma (Table...)while at the same pH only 11 % carotene is extractable from the serum albumen solution.

Alcohol has a similar liberating effect on plasma and aqueous colloidal solution of carotene. In contradiction however to the statement of V. d. Bergh and Muller that a small amount of alcohol is sufficient to cause complete extraction with ether it was found that only 65 % carotene was extracted from an aqueous solution of carotene at pH 7.3 when the concentration of alcohol was 25 %. From plasma as well as from 1 % serum albumen solution carotene is extracted completely when 50 % concentration of alcohol is reached. Quantitative differences are also observed in the effect which bile salts exert on the extractability of carotene from aqueous solution and from plasma. The reduction of the extractability is ten times greater in case of

aqueous solution than that in case of plasma, a difference best explained by supposing that carotene occurs in different forms in these two cases. Freezing produced the same effects in both plasma and aqueous solution of carotene. The machnaizm however of this phenomenon may be a quite different nature in these two cases. In case of aqueous colloidal solution of carotene it may be again a change in the dispersion of the particles while in the case of plasma disruption of a complex occurs. In 1 % serum albumen solution of carotene freezing had no effect in the one experiment performed. V.d. Bergh and Muller (5) point out themself other differences between aqueous solutions of carotene and plasma carotene. Thus, small amounts of alkali or salts of bivalent and trivalent metals added to the aqueous solution of carotene render the carotene soluble in ether or benzene, but the same substances had no effect on the extractability of carotene from human plasma or serum. There are also differences in the oxidation of carotene in aqueous colloidal,solutions and in plasma. Carotene in watery solutions undergoes complete oxidation when exposed to a quartz lamp for 15 to 90 minutes, being totally decolorised. Plasma

or serum carotene on the other hand is not affected in similar circumstances (V. d. Bergh and Muller). The protein of the complex and probably other participants of it apparently exert a protective action on the carotene. Karrer and Straus (15) have shown that lecithin and ascorbic acid have a protective antioxidising action on carotene in water solution.

There are, as may be seen, certain similarities between the form in which carotene occurs in human plasma and in aqueous solutions of this substance but the differences are too great to accept these two forms as identical. The differences between carotene in 1 % serum albumen solution and plasma carotene are smaller but great enough to reject their identity. Colloidal carotene in 1 % solution of serum albumen is probably adsorbed on the protein particle while in plasma or serum. The bond between carotene and the protein is able to undergo some change (probably according to the equation:

carotene-protein $\rightleftharpoons$ carotene + protein)

affected by various factors as well as by the concentration of the reactants in accordance to the Law of Mass Action. Unfortunately little

or nothing is known about the metabolism and the fate of carotene in human body and therefore any further analysis would be a pure speculation without experimental background. That however carotene is bound more firmly and rigidly in 1 % solution of serum albumen than in plasma may be seen from the fact that pH and freezing have litt e or no effect on carotene in 1 % albumen solution(Table 33)while in case of plasma carotene the effect of both pH and freezing is powerful.

S U M M A R Y

S U M M A R Y.

1. The extractability of carotene and vitamin A from human plasma by shaking with ether was found to be a function of pH of plasma and of the denaturation and precipitation of plasma protein.

2. Freezing of plasma with ether causes carotene and vitamin A to pass to the ether layer.

3. The existence in human plasma or serum of a complex of carotene, vitamin A and bilirubin with a serum protein (serum albumen) was confirmed.

4. Strong indications were found that cholesterol and lipoids also participate in that complex.

5. Aqueous colloidal solutions of β-carotene and solutions of β-carotene in 1 % serum albumen solution were found not to be identical with carotene in human plasma or serum.

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